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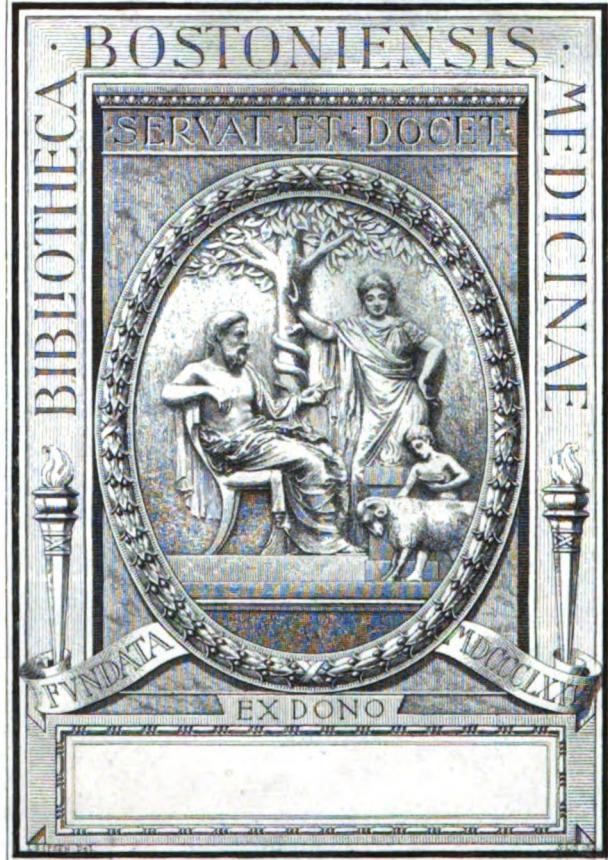
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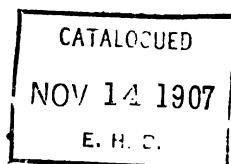
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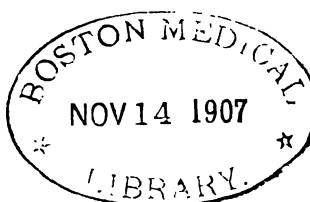
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## THE OXYGEN TENSION IN THE SUBMAXILLARY GLANDS AND CERTAIN OTHER TISSUES

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*(Received December 28th, 1905)*

At first sight it is perhaps not surprising that the various secretions of the body should be credited with a certain quantity of oxygen as well as of nitrogen. These gases are, no doubt, in a condition of simple solution and were they in the proportions in which they occur in serum their presence, on casual consideration, would not be surprising. On further investigation, however, it becomes evident that these very secretions are formed by the passage of fluid through the cells of secreting glands, these cells take up oxygen more rapidly than any other structures in the body are known to do, and therefore it is much more remarkable that such a secretion as saliva should contain as much oxygen as does the serum, than it would be if the saliva contained none. It is, however, still more remarkable that saliva and milk should be credited with containing more oxygen in solution than there is in the same volume of serum.

The present paper consists of (1) a critical discussion of the analysis of the gases in saliva, which have been obtained by previous workers, and from which it will appear that in the opinion of the author these analyses are not seriously amiss; (2) a description of some experiments which confirm them; (3) a discussion of the light thrown by the facts cited upon the physiology of certain tissues.

### I. THE OXYGEN IN SALIVA

Two physiologists have published analyses of the oxygen in saliva. The following data are given by Pflüger<sup>1</sup> :—

Quantity of saliva	Oxygen per cent.	Nitrogen per cent.
36.56 c.c.	'4	'7
33.03 c.c.	'6	'8

1. Pflüger's *Archiv*, vol. I., p. 688.

Pflüger, in discussing these figures, notices firstly that the quantity of oxygen is as great as (in the second analysis greater than) that of the plasma ; for the latter he gives the figure 5 per cent. This value for the oxygen in plasma is a purely conjectural one, for Pflüger's analysis of plasma yielded an oxygen value of 1.2 per cent. These figures were considered to be too low, since the blood when it was shed, and before the corpuscles were separated, deprived the plasma of some oxygen.

Exact determinations of the percentage of oxygen in plasma have been made by Bohr.<sup>1</sup> The maximum value given by him for the oxygen in plasma (*i.e.*, the value corresponding to a tension of 120 mm. of mercury) is 36.4 per cent. This we may take provisionally as being correct.

It appears, therefore, that both of Pflüger's analyses give an oxygen percentage in saliva which is higher than the value given by Bohr for his oxygen percentage in plasma.

An examination of Pflüger's figures does not indicate that they err on the side of being too high. Reference to his values for nitrogen shows that these are very low (7 per cent. and 8 per cent. respectively). There does not, therefore, appear to be any leakage of air into his apparatus. Indeed, his figures rather suggest that incomplete exhaustion has taken place, for 100 cubic centimetres<sup>2</sup> of water at 38° exposed to a partial pressure of nitrogen of 610 mm. (*i.e.*, 79 per cent. of an atmosphere) absorb 90 c.c. of the gas.

Passing from the figures of Pflüger to those of Külz<sup>3</sup>, it appears that the analyses performed by this author shew even higher values for the oxygen in saliva than do those of Pflüger.

The following table gives the values for oxygen and nitrogen respectively as found by Külz :—

1. Article entitled *Blutgase und Respiratorische Gaswechsel* in Nagel's *Handbuch der Physiologie des Menschen*, p. 199.

2. Bohr, *ibid.*, p. 116.

3. *Zeitschrift für Biologie*, vol. XXIII, p. 326.

Quantity of saliva analysed in c.c.	Oxygen per cent.	Nitrogen per cent.	Quantity of saliva analysed in c.c.	Oxygen per cent.	Nitrogen per cent.
100.0	1.01	2.67	24.49	1.39	3.77
24.49	1.33	2.52	25.95	0.84	2.49
25.95	1.46	3.10	100.0	0.84	2.37
25.95	1.38	3.34	100	1.25	3.20
24.49	1.19	2.65	100	1.04	2.88
25.95	1.17	2.75			

It seems probable that the above values are too high. This may be judged from the percentages of nitrogen which are given, the apparent quantity of nitrogen varies from 2.37 to 3.77 volumes per cent.

If we suppose that the percentage of nitrogen in the saliva is the same as that of the blood (1.20 according to the mean of sixteen estimations by Bohr) we can correct the figures given above. In the first column Külz gives 2.67 per cent. of nitrogen; of this 1.20 may be taken as representing the nitrogen of the saliva, and the remainder 1.47 per cent. as accidental. If, however, 1.47 per cent. of nitrogen is due to air in the pump the approximate quantity of oxygen from the same source will be one quarter of this amount, namely, .37 per cent. The amount of oxygen actually found by Külz was 1.01 per cent. It would therefore appear that the corrected oxygen in the saliva is .64 per cent (1.01---.37). This figure at all events is not likely to be too low. By applying the same correction to all Külz's figures, the following table is arrived at:—

Nitrogen recorded by Külz	Less 1.2 per cent.	Oxygen from air	Oxygen in saliva	Nitrogen recorded by Külz	Less 1.2 per cent.	Oxygen from air	Oxygen in saliva
2.67	1.47	.37	.64	3.77	2.87	.64	.75
2.52	1.32	.33	1.00	2.49	1.29	.32	.52
3.10	1.90	.48	.98	2.37	1.17	.29	.55
3.34	2.14	.54	.84	3.20	2.00	.50	.75
2.65	1.45	.36	.83	2.88	1.68	.42	.62
2.75	1.55	.39	.76				

The percentages given in the fourth and eighth columns of the above table may be regarded as minimum percentages. Two things are clear, they are of the same order as Pflügers analyses, and they all exceed the value .364 which we have taken as representing the oxygen in plasma at a tension of 120 mm.

## II. EXPERIMENTS ON THE EFFECT OF THIONIN INJECTIONS.

At a demonstration before the Physiological Congress in 1898, O. F. Grünbaum showed that if thionin were injected into the circulation and the *chorda tympani* subsequently stimulated the saliva was tinged with the bluish colour of the pigment. This observation I have repeated on three occasions, and not only so but I have found that if the submaxillary gland be cut in two while the stimulation is proceeding, the whole cut surface of the gland is a uniform blue colour. The unstimulated gland on the other side is not necessarily the same. The cut surface of the gland was almost normal in colour in two of the three experiments, whilst in the third there was just appreciably less colour than in the stimulated gland, it turns blue, however, on exposure to air. The presumption is that the thionin becomes reduced to a greater or less degree in the resting gland (as Ehrlich shewed with alizarin blue in the tissues generally). It does not get reduced either by the active gland or by the saliva. To reach the saliva, however, it must pass actually through the cells of the submaxillary gland.

In respect of its action on thionin, the submaxillary differs very obviously from muscle. During Experiment II, shortly after observing the glands, I stripped the skin off the thighs of the cat. The muscles were a little, but only a little, coloured and the same on both legs. On stimulation of the right anterior crural nerve the muscles on the anterior surface of the right thigh became obviously less coloured than those of the left side, which showed that the thionin became reduced more actively by the contracting than by the resting muscle.

The pancreas, like the salivary glands, was blue superficially and on section (Experiments II and III).

The mucous membrane of the stomach was more blue than the unstriped muscle (Experiments II and III).

The thionin was injected in normal saline (40 c.c.). The injection acted as a violent diuretic, there was a copious discharge of blue urine, after which the bladder rapidly filled and became tense with an additional secretion of a similar fluid. The kidney on section was

not blue. The natural colour, however, made it difficult to judge of any artificial pigmentation. Three hours afterwards, the section which had been cut out and which was exposed to air in the meantime, was somewhat blue in the medullary region (Experiment II).

Neither the heart nor the lungs appeared abnormal in colour (Experiments II and III).

The fat of the omentum was its usual pinkish-white colour (Experiments II and III); cut into small pieces and allowed to stand, it became slightly tinged with blue. In the case of such a tissue as fat the colouration would not be great however, as the tissue from its nature would not readily become permeated with a dye contained in aqueous solution.

The following is the protocol of the second experiment in which my original observations on the submaxillary gland were confirmed and the observation on other organs were made.

Dec. 24. Cat. A. C. E. mixture given through trachea tube cannula in femoral vein. Chorda tympani and anterior crural nerves exposed for stimulation on right side.  
12.14—12.19. 20 c.c. of solution of thionin in normal saline injected into r. femoral vein.

- 12.19. Stimulation of chorda. Good flow of saliva, blue colouration.
- 12.22. 13 more c.c. of thionin injected, chorda stimulated trace of colour in saliva.
- 12.24. 10 more c.c. of thionin injected.  
The colour was obvious in the saliva, chorda stimulated.
- 12.26. Right submaxillary cut out and cut open, whilst the stimulus was maintained.  
The gland was blue throughout.
- 12.28. Left gland (unstimulated) taken out and cut open, blue on surface, section not blue except at edge.
- 12.35. Profuse micturition, urine blue.
- 12.40. Pancreas blue on surface—stomach mucous membrane bluer than muscle.
- 12.45. Took skin off legs, same colour. Stimulated right anterior crural nerve—muscles became less blue than on left side, neither side was more than tinged.
- 12.54. Fat in omentum not coloured.
- 12.55. Bladder full of blue urine.  
4. Kidney scarcely coloured.
- 12.57. Lung and heart not coloured.
- 4.0. Specimens of left submaxillary blue, fat faintly blue, lung not coloured, kidney not obviously coloured in cortex, coloured in medulla.

Dec. 26, 11 a.m. Specimen of fat much more obviously blue.

## THEORETICAL DEDUCTIONS

After Ehrlich demonstrated the fact that alizarine blue and other stains were reduced by certain tissues, Bernstein<sup>1</sup> published a scale of the reducing powers of certain tissues. His data were derived from placing these tissues, *post-mortem*, in the pigment and studying the time necessary for its reduction. On this scale muscle appeared to have the greatest reducing power of all the organs he tested.

If the results of the experiment which I have detailed above were translated into the language of Bernstein, the following results would be arrived at :—(1) That the resting submaxillary gland had a greater reducing power than the active submaxillary ; (2) That the reducing power of the pancreas was relatively very low ; (3) That the reducing power of active glands (the submaxillary and the kidney) was very low, since the pigment which actually goes through the cells of these glands appears unreduced in the urine.

If, however, the reducing power be defined as the amount of oxygen used up per minute by one gramme of the various structures it will be seen that the following are the approximate reducing powers of the various structures mentioned above :—

Active submaxillary <sup>2</sup>	•1	...	Active kidneys	•07
Resting submaxillary	•03	...	Heart beating feebly <sup>5</sup>	•006
Resting pancreas <sup>3</sup>	•05	...	Muscles of leg resting <sup>6</sup>	•002
Resting kidneys <sup>4</sup>	•03	...	Muscles of leg tetanus*	•018

There is, therefore, an apparent divergence between the reducing power of the tissues as calculated from their oxygen-consumption and the reducing power given by Bernstein. The reason of this difference is not far to seek. Bernstein had been studying the power of reduction which *post-mortem* tissues, deprived of oxygen, possess, whilst the above figures apply to living tissues in their own environment and supplied with oxygen at the rate, and by the means, which normally satisfies their needs.

1. Schäfer's Text Book of Physiology, Vol. I, p. 782.
2. Barcroft, *Journal of Physiology*, Vol. XXVII, p. 43.
3. Barcroft and Starling, *Journal of Physiology*, Vol. XXX, p. 496, 1904.
4. Barcroft and Brodie, *Journal of Physiology*, Vol. XXXII, p. 52.
5. Taken from a research now being carried on by Dr. W. E. Dixon and myself.
6. An approximation computed from the results obtained by V. H. Mottram A. E. Stansfield, Miss Tweedie, and myself.

The colour of the tissues in my experiments with thionin cannot therefore be compared in any way with Bernstein's, nor can it be taken as a measure of the reducing power of the tissues in Bernstein's sense. It is rather an indication of the normal conditions, in respect of oxygen tension, under which these organs exist in their varying states of rest or activity. In the muscles of the leg, as in the submaxillary gland, there is an increased consumption of oxygen in the condition of activity. The active muscles become less blue, the active gland becomes more blue ; the deduction is that activity in the gland is associated with an increased oxygen tension, activity in the muscle with a decreased oxygen tension. In other words, whilst in both tissues active reduction is taking place, this active reduction is more than counterbalanced in the case of the gland by some other process which keeps up the tension of oxygen.

This conclusion is borne out by the analyses of Pflüger and of Külz, who have shewn that the fluids which have passed through the gland cells have an appreciable oxygen tension. This oxygen tension differs in the secretions of different glands, but in all it exists. The view that secreting glands carry on their work with a much more copious environment of oxygen than is given to active muscles is not a new one. It was essentially bound up with Bernard's observation that the blood from the active submaxillary was arterial in colour ; this point has been accentuated by Bohr,<sup>1</sup> who has calculated the concentration of oxygen in the submaxillary vein, during the conditions of rest and activity of the gland, from my own figures ; his calculation shews the following data for the venous blood from the submaxillary gland :—

Condition	Percentage saturation of O <sub>2</sub>	Concentration of O <sub>2</sub> in plasma
Rest ... ... ...	52.8	.089 per cent.
Chorda stimulation ...	73.3	.132 per cent.

Neither of these authors, however, have pointed out that this rise of oxygen tension is associated with an enormous oxygen consumption.

The following calculation will show the magnitude of this phenomenon.

1. *Ibid.* p. 200.

In the dog, as the result of nine experiments,<sup>1</sup> 1.4 c.c. of saliva per minute leave the gland during chorda stimulation; this is equivalent to about 1.6 c.c. of water leaving the vessels.<sup>2</sup> If the oxygen which the gland uses were transferred to the gland in the water, at the same velocity as the water of the secretion, we may ask ourselves what would be the concentration of the oxygen in the water on its journey between the blood and the cell.

The quantity of oxygen leaving the blood is (1) that used by the glands—.86 c.c.; (2) that contained in 1.4 c.c. of saliva,  $1.4 \times .007$  (mean of Külz reduced results) = .01; (3) that contained in the lymph—an unknown factor which must be neglected. The total, therefore, is .87 c.c. per minute. If this were dissolved in 1.6 c.c. of water the concentration would be 55 per cent., which would correspond to a tension of about twenty-three atmospheres of pure oxygen.

Somewhat similar calculations might be made in the cases of the kidney and the pancreas.

There are several lines of thought along which a solution of the problem before us may be sought.

In the first place, it is conceivable that, as was once supposed, the respiration of the gland takes place in the blood, *i.e.*, that the gland thrusts a reducing substance into the capillaries which directly and instantly reduces the haemoglobin. This view is opposed to all we know about tissue respiration. Further, such a substance would have to possess the property of reducing the haemoglobin in an inappreciable time without being able to break up the molecules of oxygen in the stream through which it is thrust, and thrust at a velocity greater than that of the saliva. The reason of this last statement depends upon the fact that there is free oxygen in the saliva. A body possessing a reducing power of this nature is not very remarkable, for ferrous sulphate reduces potassium permanganate instantly, whilst it only reduces free oxygen slowly. But if such a body existed, it might be expected to find its way to some extent into the saliva, and there, and also in the gland, to reduce the thionin.

1. Barcroft, *Journal of Physiology*, Vol. XXVII, p. 40.  
2. Barcroft, *Journal of Physiology*, Vol. XXV, p. 457.

Another consideration which yields a more likely solution of the problem is, that the oxygen diffuses from the blood to the gland very much more rapidly than the stream of water travels. For the passage of a given quantity of oxygen from the capillary wall to the cell, the difference in oxygen tension in the two positions necessary to produce the flux would be inversely as the specific velocity of which the molecules were capable. By supposing that the molecules passed very rapidly we might postulate a small difference of tension. But a difference of tension must exist. This difference of tension might be maintained in either of two ways : (1) by a low oxygen tension in the gland ; (2) By a high oxygen tension in the capillary wall. The experimental evidence which has been before us in this paper indicates that the former of these conditions does not exist ; we are, therefore, bound to fall back upon the conception that there is something active, some oxygen secreting quality in the capillary wall, unless we can find some other means of raising the oxygen tension in the saliva above the calculated value in the plasma.

A third possibility is that the calculated value of the tension of oxygen in the capillaries is too low—we have taken it at 120 mm. of Hg. According to the computation of Haldane this figure is much lower than the oxygen tension of the arterial blood. But the conception of oxygen tension set forth by Haldane is of no avail. For, suppose the oxygen tension of the arterial blood to be 160 mm.—true the concentration of oxygen in the plasma would rise to a value approaching that given by the analysis of saliva—but the dissociation-curve of oxy-haemoglobin is such that the tension would at once fall when the first vestige of oxygen left the haemoglobin.

Adhering to the conception that the calculated oxygen tension in the capillary is too low, we might suppose that some constituent of the blood played the role of actually turning the oxygen out of the haemoglobin in the capillaries.

Bohr has brought forward evidence of  $\text{CO}_2$  performing such a function, but on far too small a scale to account for the phenomenon which we are considering. We know of no other substance which can be held to perform this function in the blood.

## CONCLUSIONS

1. The analysis of Pflüger and Kulz show that the oxygen tension in saliva is greater than that in plasma, and that there is a definite oxygen tension in other secretions, milk, urine, etc.
2. Injection of thionin shews that the active submaxillary gland does not reduce the dye sufficiently to prevent the gland becoming blue throughout. The saliva and the urine are also blue.
3. The salivary gland becomes bluer during activity, the muscles of the leg less blue.
4. In view of the great consumption of oxygen in the submaxillary gland, the high oxygen tension of saliva is not easy to account for, the most probable of the explanations discussed is that the capillary walls have the power of raising the tension of the gas as it passes through them.

# A METHOD FOR DETERMINING THE TOTAL DAILY GAIN OR LOSS OF FIXED ALKALI, AND FOR ESTIMATING THE DAILY OUTPUT OF ORGANIC ACIDS IN THE URINE, WITH APPLICATIONS IN THE CASE OF DIABETES MELLITUS

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A large number of methods have been published for determining and estimating the reaction of the urine.

The number of such investigations is so large that individual reference to the several papers and an exhaustive review of the literature becomes impossible, but it may be stated that the figures vary enormously according to the method and the indicator used for the purpose.

In addition to the experimental difficulties introduced by the fact that the urine is itself coloured, and hence interferes with the delicacy of the reaction to coloured indicators, there is the more important fact that the reaction of the urine is never due to free acid or to free alkali, but to a varying mixture of salts such as the primary and secondary phosphates of the alkalies. In such a mixture the urine reacts entirely differently to different indicators. Thus the same sample of normal urine is *acid* to a sensitive indicator to weak acids such as phenol-phthalein and *alkaline* to a stable indicator such as methyl-orange or di-methyl-amido-azo-benzol. Also if the titration figures to three such indicators as phenol-phthalein, litmus, and 'di-methyl' be taken, it will be found that there is a high acidity with the phenol-phthalein, a much lower acidity with the litmus, and a high alkalinity with the di-methyl indicator. When similar titrations are undertaken in the case of an artificial mixture of the primary ( $\text{Na}_2\text{H}_2\text{PO}_4$ ) and secondary ( $\text{Na}_2\text{HPO}_4$ ) phosphates of sodium in water to which such phosphates have been added in known amount and ratio,

the reason underlying the differences in the titration values becomes at once apparent. The neutral point for phenol-phthalein lies at the point where the kations and anions are so distributed as to correspond to  $\text{Na}_2\text{HPO}_4$ , while for di-methyl (or methyl orange) the neutral point corresponds to  $\text{Na}_2\text{H}_2\text{PO}_4$ , and for litmus the neutral point lies somewhere intermediate between these two values. It is clear from this statement that it is futile to regard any one indicator as the arbiter of neutrality, and to consider a solution as being neutral because it is neutral to phenol-phthalein when it is alkaline to litmus and 'di-methyl,' or when it is neutral to litmus or laemoid, and acid to phenol-phthalein and alkaline to di-methyl at the same time.

The only true definition of neutrality would be the point at which the concentrations of hydrogen and hydroxyl ions are equal, and no coloured indicator satisfies this condition but indicates neutrality at a point where there is a given ratio other than equality between the acidic and basic ions. The value of this ratio depends on the ease with which the coloured indicator, and its ions, associate or dissociate in solution.

The proper method of determining reaction ought, therefore, to be some method of determination of the concentration of hydrogen or hydroxyl ions which would indicate where these two concentrations were equal.

In the case of a solution of the mixed phosphates, such as the urine, all physical methods for determination of the ionic concentrations fail, however, in accuracy on account of the very slow variation of the concentration in the two ions around the neutral point. In the case of free acid or free alkali, the smallest addition of acid or alkali to the solution in the neighbourhood of the neutral point causes an immense swing in the ratio of the two ions which is at once obvious in the gas battery; whereas, in the case of a solution containing phosphates, the degree of dissociation is low, and an addition of acid or alkali causes not a great swing in the ratio of hydrogen or hydroxyl ions but a decomposition of phosphate in either direction, and the establishment of a new equilibrium in which the ratio of the two ions may not be widely different from the former.

On this account a solution such as the urine, or any of the body fluids in general, behaves as a controlling agent or as a neutralizing agent for either acid or alkali, and prevents large variations in either hydrogen or hydroxyl ion concentration.

The importance of such a regulating mechanism to the organism, the cells of which are so sensitive to such variations, is too obvious to need elaboration.

It is also the slow variation in the ratio of hydrogen and hydroxyl ionic concentration as acid or alkali is added which renders the change in colour, when coloured indicators are used, more gradual than in the case of free acid or alkali and causes a difficulty in determining the exact point of change.

Returning to the indications given by coloured indicators in the case of such physiological fluids as urine, it may be stated that the neutral point to each colored indicator means a different equilibrium point between acid and basic ions. Accordingly, the description of such points as neutral points may lead to serious error and misunderstanding unless it be remembered that neutrality means in such a case an equilibrium with a certain ratio of the two ions as shown by a certain indicator and not an equality in the two ionic concentrations.

With this safeguard, however, valuable information can be often obtained as to the distribution of the two phosphates (or rather of the kations and anions) in the solution, provided indicators be used which react and show their change in colours at definite points, such as phenol-phthalein and methyl-orange or di-methyl, and not those which change at intermediate points as litmus.

While this can be carried out in the case of urine at the phenol-phthalein end showing the amount of alkali necessary to be added to reach the equilibrium point corresponding to  $\text{Na}_2\text{HPO}_4$ , it cannot be accurately carried out at the other end with a stable indicator so as to give the amount of acid necessary to be added in order to reach the equilibrium point  $\text{NaH}_2\text{PO}_4$ , on account of the yellow colour of the urine.

There is another and physiologically more important circumstance which renders it impossible to determine by any combined use of

coloured indicators whether acid or alkali is gained or lost to the organism by the channel of the urine.

The organism protects itself from any variation in the ratio between the hydrogen and hydroxyl ionic concentrations to which its cells are habituated by the formation of organic acids, or organic bases (or ammonia), which combine with and neutralize the excess of base or acid and carry it off in neutral form in the urine.

The mere determination of the alkalinity or acidity of the urine gives on this account no real idea of how the balance is being varied between acid and alkali in the body by the action of the kidneys.

It was with the object of discounting in some manner this action of the tissue cells in producing, according to circumstances, organic base and ammonia, or organic acid, and so disguising the real output of fixed alkali or acid, that the methods of determination described in this paper were devised.

The real output of fixed alkali or acid having been once determined, one obtains incidentally, as will be shown later, by comparison with determinations (*a*) of the apparent output as given by the reaction of the urine, and (*b*) by separate determinations of the ammonia, a means of estimating the amount of organic acid or organic base manufactured daily by the body in order to maintain the ratio between hydrogen and hydroxyl ions existing in the body.

This latter determination is of high importance in pathological conditions where factors are at work tending to disturb the balance in the tissue fluids between the two ions, as for example in the large manufacture of organic acids which occurs in diabetes.

Before passing to the description of the details of our methods, we may give one or two illustrations of the fact that the reaction of the urine, determined in the ordinary way, gives no real estimate of the ratio between the amount of acid and alkali being carried off from the body in the urine.

It is proverbial in therapeutics how difficult it is to alter the reaction of the urine. It is only when enormous doses of alkali, in the form of alkaline carbonates, are given that the reaction of the urine becomes alkaline to litmus, and, again, large doses of mineral

acids can be given without appreciably altering the reaction of the urine to litmus. Here the alkali or acid, as the case may be, must leave in some form in the urine, yet the reaction of the urine gives us scarcely any indication of the process.

The explanation is, of course, an easy one, the body in the case where acids are given forms a base (such as ammonia or an organic base), and in company with this, the acid is carried off in the urine, or when alkali is given, less ammonia or organic base is formed in order to leave acid for combination with the alkali, or, if this is insufficient, more organic acid than normal is formed. It is only when the power of these regulatory mechanisms has been surpassed that obvious changes begin to take place in the reaction of the urine, and at about this level acid or alkaline intoxication begins to intervene.

Similar carriage of acid or alkali away in the urine may occur without any indication appearing in the reaction of the urine in cases where the acid or alkali is not given by the mouth, but appears as a result of pathological changes within the organism itself. For example, there is no indication in the reaction of the urine of the amounts of organic acid which are carried off in cases of diabetes. Also when the production of such organic acids exceeds a certain limit, and ammonia can no longer be produced in sufficient amount to neutralize them, a drain commences upon the fixed alkalies of the body, and sodium salts of the organic acids begin to be secreted in the urine. It is at this level that the amount of alkali in the blood becomes reduced and acid intoxication and coma are threatened ; yet we possess no signal in the reaction of the urine to give definite warning of the commencement of such a condition of affairs.

An appreciation of the importance of determining the amount of organic acid leaving the body in diabetes is seen in the many attempts that have been made to obtain methods for estimating the amounts of each of the organic acids found in the urine in diabetes.

The general fact appears to have been lost sight of in devising these separate methods for the estimation of the amount of each acid, that the acids concerned are not poisonous or dangerous on account of any special toxic properties attached to any special group in their

molecule, but merely as so much acid which is capable of neutralizing and carrying of alkali, and so causing acid intoxication. They are organic acids which the diabetic has lost the power of oxidizing, they must be neutralized by alkali, and therein lies their danger.

Hence, what is required, is not a method for estimating each acid separately, but a method for estimating the total output of organic acids in the urine, and at the same time for indicating whether the organism is still capable of meeting this output and neutralizing it by ammonia, formed in lieu of so much urea which would have normally been produced, or whether the fixed alkali of the blood and tissues is being utilized and drained away from the body.

Such a method we have devised and carried out with the results shown in the protocols of our determinations.

The first step in the process is the determination of the excess of fixed inorganic acid or alkali leaving the body daily.

This is determined by adding excess of standard alkali to a measured volume of the urine, incinerating, adding excess of standard acid and boiling, in order to re-convert back into orthophosphates any pyro- or meta-phosphates which had been formed in the process of incineration, and then titrating to neutrality. The difference in the total amount of standard alkali and acid used then gives the loss or gain in alkali to the body through the urine. The excess of alkali is added to prevent loss of inorganic acid combined with ammonia or organic base in the process of incineration. In the incineration any fixed alkali combined with organic acids is converted into carbonate and hence appears in the difference between the amounts of standard alkali and standard acid used. Accordingly the figure obtained gives the difference between inorganic acid and inorganic base (minus ammonia) in the urine. Since the ammonia and organic acids and bases of the urine may be regarded as formed in the body, the figure obtained shows whether excess of acid or excess of alkali, other than that formed in the body, is escaping daily by the urine. Thus, if more of the standard alkali than of the standard acid is required to give neutrality in the incinerated urine, then acid is leaving in excess, and *vice versa*.

The figure so obtained gives those variations which ought naturally to follow administration of alkali or acid, a figure which is

never obtained by direct titration of the urine. Thus, in Case II, Table II, it is seen that when sodium bicarbonate is administered less alkali than acid is required in order to obtain neutrality, although the unincinerated urine is still acid.

Also, the decrease in the figure of alkali added *minus* acid added, indicates in pathological cases, such as diabetes, a commencing drain upon fixed alkali, or in other words that the body is commencing to fail to turn out enough ammonia to neutralize the organic acids.

In order to obtain the total amount of organic acids formed, two additional determinations are required, these are (1) the acidity of the urine without previous incineration in the ordinary way, and (2) the amount of ammonia.

In making the determinations of neutrality of the urine before and after incineration the same indicator must be employed in the two cases, in order to leave the balance between the inorganic ions at the same level in the two cases. In making the determinations of ammonia for which we have employed the Schlössing process, any indicator may be used which is sensitive to ammonia and gives the full yield—the indicator used by us was di-methyl-amido-azo-benzol. On account of the yellow colour of the urine we have found phenol-phthalëin the best indicator to use for the two titrations in the urine, and having found that when one proceeded from the acid side towards neutrality there was no appreciable error introduced by using phenol-phthalëin even in the presence of ammonia, we have used this indicator throughout.

Great care was taken to check this before committing ourselves to the use of phenol-phthalëin as an indicator in presence of ammonia ; both by titrating dilute ammonia solutions of known strength, by first adding excess of standard acid, and then titrating back in presence of phenol-phthalëin, and by comparing titration results with the same solution, using in one case phenol-phthalëin, and in the other di-methyl. The results were always closely concordant, although the change in colour is much less sharp than in the case of fixed alkalies.

If, however, the titration is carried out from the acid side with standard alkali, and the first faint appearance of pink is taken as the

sign of neutrality, phenol-phthelëin indicates the amount of ammonia quite accurately. If the phenol-phthalëin be added in presence of free ammonia and titration with acid be attempted there is, however some effect of the ammonia upon the indicator and wrong results are obtained.

In urine which may contain a great deal of ammonia, however, the reaction is always acid to phenol-phthalëin, and hence direct titration with alkali can always be undertaken without any interference or error due to the presence of the ammonia.

The manner in which the three determinations mentioned above, yield an estimate of the excess of organic acid above organic base formed in the body may be illustrated by giving a formal statement of what is determined in each process, as follows :—

I. Direct titration with  $\frac{N}{10}$  alkali = Acid salts of inorganic acids + acid salts of organic acids + free inorganic acids + free organic acids.

II. Total  $\frac{N}{10}$  acid added — total  $\frac{N}{10}$  alkali added in incineration process  
(i.e. total alkali produced by incineration) = Organic acids combined with fixed bases—  
free inorganic acids — ammonium salts of inorganic acids — acid salts of inorganic acids.

III. Total ammonia = Ammonium salts of organic acids + ammonium salts of inorganic acids.

On addition, I + II + III = Acid salts of organic acids + free organic acids + organic acids combined with fixed bases + ammonium salts of organic acids.

That is to say, addition of Nos. I, II, III give the total organic acid, combined or free in the urine, and all the other constituents cancel out.

In the scheme above, for completeness in statement, free inorganic and free organic acids are included although these are not usually present, if absent, however, these constituents simply drop out of the scheme, and the addition still gives the total organic acids.

Nos. I and III in the above equations are so obvious as to require no comment ; regarding No. II it may be pointed out :—(1) Any organic salts of fixed bases are converted into carbonates on incineration, and so increase No. II ; (2) any organic salts of ammonium or organic bases disappear on incineration, and so do not affect No. II ; (3) any inorganic ammonium salts are converted into corresponding sodium salts, and so use up alkali and decrease No. II ; (4) any free inorganic

acid or inorganic acid salts require alkali for neutralization, and so decrease No. II.

Before passing to the tables of results, the procedures and precautions in carrying out each of the three determinations enumerated above may be described.

I. *Initial titration of the urine.* Twenty c.c. of urine are taken and diluted to about 100 c.c. in order to prevent the urinary pigments from obscuring the end point of the reaction. On account of the change of colour with phenol-phthalëin being more obvious than that with other indicators in a solution of urine, and of its sensitiveness to organic acids, this indicator has been employed. The titration is made with  $\frac{N}{10}$  sodium hydrate solution, and the first appearance of pink is taken as the end point.

II. *Titration after incineration.* Fifty c.c. of urine are taken, and 10 c.c. of N sodium hydrate solution are added, so as to give a considerable excess of free alkali with the object of preventing any loss of acid which may be present in the urine in combination with ammonia or organic bases. The mixture is evaporated to dryness in a platinum vessel, and incinerated at a low red heat. The ash is extracted with boiling water, and any unconsumed carbon is filtered off. The filtrate and washings are roughly titrated with  $\frac{N}{10}$  sulphuric acid, with phenol-phthalëin as indicator, and an excess of about twice the amount of acid necessary for neutralization is added. The solution is then boiled for about fifteen minutes in order to remove carbonic acid, and to reconvert back into orthophosphates any pyrophosphate which may have been formed in the process of incineration. The final stage in the operation is the titration back to neutrality with  $\frac{N}{10}$  sodium hydrate. The difference between total acid added and total alkali gives the figure for determination No. II. The reason for adding such a large excess of acid after incineration is that it is necessary to have free acid present, because the acid phosphates are so little ionized that the process of conversion into orthophosphates in their presence alone takes a long time, and may be incomplete even at the end of several hours boiling ; in the presence of free acid the conversion takes place rapidly, and is complete in a few minutes.

III. *Determinations of total ammonia.* These were made by Schlössing's method, using 25 c.c. of urine, and taking care that the layer of urine and lime was shallow so as to afford a large surface. If this precaution be neglected, the total amount of ammonia is not obtained even in a week's time.

### RESULTS WITH ARTIFICIAL MIXTURES

The method was tested in artificial mixtures containing phosphates, ammonium salts, and organic acids, with the results shown below, and was then applied to normal urine, and subsequently to three cases of diabetic urine for long periods.

*Mixture No. 1.* A solution was prepared containing the following constituents in the amounts stated per litre :—Sodium chloride, 6 grams; ammonium chloride, 1.15 grams; primary sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 2.0 grams; acetic acid, 340.8 c.c. of  $\frac{N}{10}$ . The mixture was analysed as above described with the following results, expressed in amounts per 1,000 c.c. of the mixture :—

	Acidity of mixture expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise 1000 c.c. of mixture	Alkali produced on incineration, that is acid added — alkali added in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Ammonia in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Organic acids expressed in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture
Calculated	—	—	215	340.8
Determined	484	— 344	204	344.0*
* c.c. 484 + 204 — 344 = 344				

*Mixture No. 2.* A solution was prepared containing the following constituents in the amounts stated per litre :—Ammonium chloride, 4.61 grams; primary sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 2.0 grams; secondary sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), 2.0 grams; sodium acetate, 3.2 grams; urea, 8.0 grams.

The results of the analysis were as follows :—

	Acidity of mixture expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise 1000 c.c. of mixture	Alkali produced on incineration, that is acid added — alkali added in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Ammonia in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Organic acids expressed in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture
Calculated	—	—	863	234
Determined	168	— 770	828	226*
* 168 — 770 + 828 = 226				

*Mixture No. 3.* Solution contained in each litre, ammonium chloride, 3.07 grams; primary sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 2.0 grams; acetic acid, 676.0 c.c. of  $\frac{N}{10}$

	Acidity of mixture expressed in c.c. of $\frac{N}{10}$ alkali required to neutralize 1000 c.c. of mixture	Alkali produced on incineration, that is acid added - alkali added in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Ammonia in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture	Organic acids expressed in c.c. of $\frac{N}{10}$ per 1000 c.c. of mixture
Calculated	—	—	575	676
Determined	818	- 694	552	676*

\*  $818 - 694 + 552 = 676$

These three experiments, and others which we have not recorded, show clearly that the method yields accurately the amount of organic acids in a solution such as the urine.

### DETERMINATIONS IN NORMAL INDIVIDUALS

No.	Daily output of urine in c.c.	Total daily acidity expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise	Total daily alkali produced on incineration, that is, acid added - alkali added in c.c. of $\frac{N}{10}$	Total daily ammonia expressed as c.c. of $\frac{N}{10}$	Total daily organic acids expressed as c.c. of $\frac{N}{10}$
1	2280	364.8	- 656.6	428.6	136.8
1	1840	504.2	- 1295.4	368.0	- 423.2
1	1850	303.4	- 329.3	488.4	462.5
1	1930	289.5	- 482.5	409.2	216.2
1	1280	371.2	- 440.3	397.4	328.3
2	1620	366.1	- 602.6	197.6	- 38.9
2	1410	400.4	- 747.3	186.1	- 160.8
2	1020	357.0	- 767.0	444.7	34.7
2	1650	343.2	- 402.6	244.2	184.8
2	1210	350.9	- 363.0	406.6	394.5

The negative figures in the third column show that inorganic acid in excess of base leaves the body daily, and indicate the amount of such excess. Attention may also be drawn to the variation in output of organic acid, even in normal individuals. A negative figure in the final column indicates that the excretion of organic bases other than ammonia is in excess of the excretion of organic acids.

The most probable cause of the variations in health shown in the table is a variation in the amount of acid or base in the diet of the individual, the variation in organic acid excreted expressing the action of the organism in maintaining a normal reaction in its media.

DETERMINATIONS IN DIABETIC PATIENTS<sup>1</sup>

The amount of fixed alkali leaving the body daily and the output of organic acids was estimated in three cases of diabetic patients.

In the first case this was done daily throughout a period of six months, an abstract of the results only is given in the tables, containing periods illustrative of special points.

Case I. Male, aged 25. In this case Table I shows effects upon alkali, and organic acid output of administration of alkaline carbonates (sodium bicarbonate, one drachm (about four grammes) three times daily); Table II shows the changes in amount of ammonia and organic acids when not under continued administration of the alkali, and also illustrates the effects of a small dose of alkali occasionally under such conditions, the days on which alkali in the form of a seidlitz powder was administered being shown by an asterisk.

CASE I. TABLE I

Date 1904	Daily output of urine in c.c.	Total daily acidity expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise	Total daily alkali produced on incineration, expressed as c.c. of $\frac{N}{10}$	Total daily ammonia expressed as c.c. of $\frac{N}{10}$	Total daily organic acids expressed as c.c. of $\frac{N}{10}$
Nov. 1	3400	1029	1224	156	2400
" 3	3580	1647	788	165	2600
" 5	3700	1332	2057	89	3478
" 7	3980	1274	2579	80	3933
" 9	3980	613	3025	64	3702
" 11	3690	517	1727	118	2362
" 12	3410	423	1610	102	2135
" 14	1700	228	442	228	898
" 17	3520	493	1422	77	1992

At this stage the administration of bicarbonate (3 drachms or about 12 grams daily) was stopped

Nov. 19	3350	737	951	121	1809
" 21	2560	589	430	256	1275
" 23	2270	658	354	308	1320
" 25	2380	643	286	409	1338
" 27	2440	791	312	386	1489
" 29	1190	357	55	643	1055
Dec. 1	1360	245	76	530	851

Patient left hospital.

<sup>1</sup>: The character of the cases is given in the succeeding paper, pp. 28 to 38.

In this table it is to be noted that the alkali produced on incineration gives a positive figure throughout on account of the large amount of alkaline carbonate being administered. Contrast with Table II, Case I, where only an occasional seidlitz powder was being given, and also with Tables I and II, Case II. The presence of this amount of base in the urine is entirely concealed by the formation of organic salts, as is shown by the fact that the reaction of the urine remains acid throughout, although there is a decrease in the amount of acidity. It is also to be noted that during the alkaline carbonate administration the amount of ammonia is very low.

On stopping the administration of alkaline carbonates at the point shown in the Table, there commences a drop in the alkalinity on incineration which falls almost to zero, and, no doubt, would soon have shown a negative value as in Table II, or in Case II, Table II. Also there is a corresponding drop in organic acids which soon reach a steady value, lying much below that found during the administration of the alkaline carbonate. The amount of ammonia shows a great increase when the alkaline carbonate treatment is discontinued.

CASE I. TABLE II.

1905 Date	Daily output of urine in c.c.	Total daily acidity expressed in c.c. of N 10 alkali required to neutralise	Total daily alkali produced on incineration, expressed as c.c. of N 10	Total daily ammonia expressed as c.c. of N 10	Total daily organic acids expressed as c.c. of N 10
Jan. 10	2220	1021	- 1732	1763	1052
" 11	1850	932	- 1513	1121	540
" 12*	2500	1150	- 1690	870	330
" 13	1900	1026	- 125	1186	2087
" 14	2270	1008	- 617	1348	1739
" 15	2160	1382	- 1378	920	924
" 17	2190	1117	- 644	907	1380
" 19	2190	1082	- 1310	639	411
" 20*	2100	1079	- 1403	810	486
" 22	1480	1146	- 941	616	821
" 23	1760	1169	- 190	553	1532
" 24	2020	1180	- 170	1709	2719
Feb. 12	1730	1263	- 1547	827	543
" 13*	1870	1384	- 755	838	1467
" 14	2100	1604	- 227	848	2225
" 15	1530	1352	- 1493	927	786

In this table it is to be noted that the ordinary titration figure gives no indication of the administration of the alkali on the days marked by the asterisks. This is shown clearly, however, by the incineration figure in the next column, the drop in the negative value showing increased amount of base in the urine after these days. When the administration of alkali is continuous as in Case I, Table I, and in Case II, Table II, the negative figure after incineration becomes a positive one, showing the increased excretion of base under alkali administration. It is also to be observed that the amount of organic acid excreted becomes increased after the alkali, and then drops, to be again increased by the next dose of alkali. Taken together these facts indicate that the alkali given is neutralized in the body by organic acids, and that the body is protected from alkaline invasion in this way by increased formation of organic acids. This relationship is worth remembering in all attempts to prevent the effects of organic acids in the blood by means of administration of alkaline carbonates.

Case II. Male, aged 7. Table I shows alkali and organic acid output under strict diabetic diet. Table II shows effects upon alkali output of administration of alkaline carbonates, and, in the latter part, the effect of stopping the carbonates.

CASE II. TABLE I.

Date 1905	Daily output of urine in c.c.	Total daily acidity expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise	Total daily alkali produced on incineration, expressed as c.c. of $\frac{N}{10}$	Total daily ammonia expressed as c.c. of $\frac{N}{10}$	Total daily organic acids expressed as c.c. of $\frac{N}{10}$
July 4	1110	546	- 107	706	1145
," 7	1820	510	- 29	903	1384
," 9	1730	467	- 367	837	937
," 12	1700	432	- 394	1129	1167
," 14	1480	524	- 255	1154	1423

## CASE II. TABLE II

Effect of administering alkaline carbonates.

Date 1905	Daily output of urine in c.c.	Total daily acidity expressed in c.c. of $\frac{N}{10}$ alkali required to neutralise	Total daily alkali produced on incineration, expressed as c.c. of $\frac{N}{10}$	Total daily ammonia expressed as c.c. of $\frac{N}{10}$	Total daily organic acids expressed as c.c. of $\frac{N}{10}$
July 16	1480	397	512	929	1838
" 19	1990	418	704	788	1910
" 21	2160	432	1041	691	2164
" 23	2160	410	1961	311	2682
" 26	2170	87	2226	286	2599
" 28	1740	14	1688	230	1932
" 30	2000	48	1344	336	1728
Aug. 2	1770	35	1685	287	2007
" 6	1650	50	1432	310	1792
" 10	1980	20	1552	143	1715
" 13	2510	40	1852	251	2143
" 18	2220	-9	1332	195	1518

At this point the administration of alkaline carbonates was discontinued.

Aug. 20	1310	327	-576	356	107
" 22	1280	338	-867	630	101
" 25	1420	327	-747	517	97
" 29	1030	361	-758	499	102
Sep. 3	1340	322	-825	504	1
" 8	1190	309	-369	367	307
" 12	1080	324	-577	373	119

In Table I it is seen that the alkalinity on incineration gives a negative figure, while the ammonia is high and the organic acids much higher than normal (contrast tables for normal individuals). Under the influence of the alkaline carbonates the alkalinity on incineration acquires a high positive value. The amount of alkali is so great that the reaction of the urine decreases in acidity and finally becomes faintly alkaline, but the change is not nearly so great as that shown by the incineration figures. Corresponding to this there is a marked increase in the amount of organic acids secreted. Finally, on stopping the alkaline carbonates, the alkalinity on incineration becomes a negative figure again, the ammonia increases again, though not back

to its former level, and the amount of organic acid drops not merely to its former level but down to a normal figure. The changes in ammonia and organic acid towards the end was not due solely to stoppage of the alkaline carbonate, but to another factor, *viz.*, treatment by acid extract of duodenal mucous membrane, which also had the effect of causing a disappearance of sugar, as explained in the succeeding paper (pp. 28 to 38).

Case III. Female, aged 9. This case was not put upon strict diabetic diet, and the quantities are from 8 p.m. to 8 a.m. daily. So that for comparison with the other tables they should be approximately doubled.

CASE III. TABLE I

Date 1905	Half-daily output of urine in c.c.	Total half-daily acidity expressed as c.c. of $\frac{N}{10}$ alkali required to neutralise	Total half-daily alkali produced on incineration, expressed as c.c. of $\frac{N}{10}$	Total half-daily ammonia expressed as c.c. of $\frac{N}{10}$	Total half-daily organic acids expressed as c.c. of $\frac{N}{10}$
Nov. 16	850	252	-29	133	356
„ 21	680	292	301	291	884
„ 24	650	266	241	471	978
Dec. 5	540	268	-11	272	529

## CONCLUSIONS

1. A method is described for determining the output of fixed alkali or acid from the body, and also for determining the total output of organic acid.
2. It is the total output of organic acid in diabetes which is of highest importance in relationship to acidosis, or acid intoxication, and not the nature of the individual acids concerned.
3. Administration of alkali, while it yields base for neutralization of organic acids in the body and so decreases the amount of ammonia, at the same time markedly increases the output of organic acids as salts.
4. This property illustrates the mode in which the body normally protects itself from alkaline invasion.

When alkali is given, unless certain limits are passed, the urine remains acid, the alkali being neutralized by organic acid formed, but incineration shows that fixed alkali is now leaving the body, as organic salts.

This is interesting as forming a parallel to the somewhat similar mode of protection against acids. In the case of acids, urea is converted into ammonia which is used to neutralize the acid, and the ammonium salts can then be excreted without appreciably changing the reaction of the urine. In the case of alkalies, organic acids are formed from carbohydrate, and used to neutralize the alkali, and as before these can be excreted without appreciable change in the reaction of the urine. In this connection an interesting fact is the larger amount of organic salts in herbivorous urine.

5. The reaction of the urine, without previous treatment, accordingly gives no real indication of the amount of fixed acid or alkali leaving the body. The variations in output of organic acids in health are considerable, and probably express the reaction of the organism to variations in the amount of acid or base in the diet, whereby a normal reaction is maintained in the body fluids.

## ON THE TREATMENT OF DIABETES MELLITUS BY ACID EXTRACT OF DUODENAL MUCOUS MEMBRANE

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The carrying out in the Bio-Chemical Laboratory of the experiments on the secretion of organic acids in diabetes described in the previous paper supplied an opportunity for testing the hypothesis that the *internal* secretion of the pancreas might be stimulated and initiated (similarly to the external secretion) by a substance of the nature of a hormone or secretin yielded by the duodenal mucous membrane; and that in certain cases of diabetes the appearance of sugar in the urine might be due to functional disturbance occasioned by the absence of such an intestinal excitant of the internal secretion.

The fact that an internal secretion of the pancreas is necessary for the regulation of carbohydrate metabolism, and that in the absence of this secretion oxidation of dextrose cannot be carried out, with the result that death occurs from acute diabetes, has been clearly proven by the experiments of Minkowski and v. Mering,<sup>1</sup> and others on complete and partial pancreas removal, and the history of the subject is too well-known to require detailed description here.

It was suggested by Schäfer that the formation of the internal pancreatic secretion might possibly be due to peculiar nests of cells of different form and staining properties to the other secreting cells of the gland.

These cells from their isolated appearance, and after the name of the observer who first described them, are called the 'islets of Langerhans.'

1. *Archiv. of Exper. Path. u. Pharm.*, 1890, Vol. XXVI, p. 371; Minkowski, *Arch. of Exper. Path. u. Pharm.*, 1893, Vol. XXXI, p. 85.

More recently, however, it has become doubtful whether these peculiar cells have in reality any special connection with the internal secretion of the gland, for it has been shown by Dale<sup>1</sup> that after prolonged excitation of the external secretion of the gland by means of secretin, there is a great increase in the number of the islets of Langerhans, from which it would appear most probable that the islet condition is only a phase into which the normal secreting pancreatic cells pass as a result of activity.

It will be shown later in this paper that the solution containing secretin at the same time that it stimulates the external secretion also probably stimulates the internal secretion, so that the hypothesis is not quite ruled out that the prolonged chemical stimulation of the cells of Langerhans may have been responsible for the changes observed in the islets.

The more probable hypothesis is, however, that the pancreas contains but one type of secreting cell which yields both the internal and external secretion, and that the cells of the islets of Langerhans are ordinary pancreatic cells in a phase of exhaustion.

If this be the case the likelihood is increased that anything which stimulates the external secretion will also stimulate the internal secretion which passes either directly or indirectly by the lymphatics to the blood stream.

It has been clearly shown by Bayliss and Starling,<sup>2</sup> that the activity of the pancreas as far as its external secretion is concerned is normally called forth by chemical agency by means of a substance termed by these authors *secretin*, which is formed by the cells of the duodenal mucous membrane and carried to the pancreatic cells in the blood stream.

Bayliss and Starling were also able to prepare by treatment of the scraped off mucous membrane by dilute hydrochloric acid, a solution containing the active substance, secretin, which called forth most powerfully an external secretion from the gland on being injected into a peripheral vein.

1. *Proc. Roy. Soc.*, 1904, Vol. LXXIII, p. 84.

2. *Proc. Roy. Soc.*, 1902, Vol. LXIX, p. 352; *Jour. of Physiology*, 1902, Vol. XXVIII, p. 325; *Ibid.*, 1903, Vol. XXIX, p. 174.

The active material was also shown to be capable of withstanding boiling so that it is not a coagulable proteid, and other properties of the active substance were worked out by W. A. Osborne.

Now if the view be taken that the cells of the pancreas are all of one type it is very probable that in the process of excitation of the external secretion by secretin, the internal secretion is also involved, and even if the functions of internal and external secretion by the gland be regarded as distinct and separate processes, it becomes highly desirable to test whether the duodenum does not also supply a chemical excitant for the internal secretion of the pancreas.

This line of argument appears to have occurred to the discoverers of secretin themselves, for Starling mentions a case of diabetes which was tested by Spriggs by injections of secretin solutions but with negative results.

It would, however, be illogical to rule out the hypothesis outlined above upon the evidence of one negative case, or even a number of negative cases of treatment of diabetes with secretin. For the position is one which can be proven by a certain percentage of positive results even if negative results occur alongside.

If, for the purpose of argument, we take it that the duodenum does yield a chemical excitant for the internal secretion of the pancreas, and that in the absence of the internal secretion glycosuria results, then there are three places in the chain at which weakness due to functional or other disarrangement may occur and lead to a breakdown and the appearance of diabetic conditions. First, the breakdown may occur at the duodenum, on account of the non-secretion of the excitant ; secondly, the breakdown may take place at the pancreas, so that although the excitant is formed at the duodenum and carried to the pancreas, yet these cells are not capable of excitation, either from complete morbid change or from some functional alteration in their metabolism ; and thirdly, there is the possibility, that even when the duodenum is normal and supplying its excitant, and although the pancreas is also normal and yielding, as a result of the action of the excitant, its internal secretion, yet there are changes in the oxidizing tissues such as the liver or muscles which prevent the oxidizing function of these from coming into operation.

It is clear that it is only in the first class of case that benefit might be expected to follow in a diabetic from administration of extracts of duodenum, even granting that the experimental difficulties of administration had been so overcome that the active material entered the circulation and reached the pancreas as if it had naturally been formed in the patient's duodenum.

Accordingly, it is scarcely to be expected that in all cases administration of extracts of duodenal mucous membrane will cure, or even benefit, diabetics, and to prove the existence of a specific chemical excitant for the internal secretion of the pancreas formed in the duodenum, it is only necessary to show in a fair percentage of cases that abolition of glycosuria follows administration of the extract of duodenal mucous membrane.

The three cases recorded in this paper form a commencement in this direction, and, although the number of cases is small, the results are promising, and we publish them in order to attract attention to the subject, and have the matter tested by other observers in a larger number of cases, premising that positive results cannot, for the reasons given above, be expected in all cases.

Before passing to the description of the cases, it may be pointed out that the view that diabetes may, in a certain percentage of the cases, arise primarily at the duodenum and not at the pancreas, may, if substantiated, cast light upon some of the *post-mortem* findings in diabetes.

Although in a certain number of cases gross lesions are found in the pancreas (put down by some authorities at about 30 per cent. of the cases), yet, in the majority of cases, no such lesions are discoverable, and the gland, both macroscopically and microscopically, is to all appearances normal. Nor has any causal connection with diabetes been found in the condition of the islets of Langerhans, which are, in the majority of cases, found with their normal appearance. A percentage of such cases, at least, of normal pancreas associated with diabetes, may find their explanation in the fact that the mischief lay in the duodenum.

*Method used for preparing the extract.* The upper three or four feet (about one metre) of the small intestine of the pig, obtained fresh from the abattoir, is taken, and laid open from end to end. The mucous surface is then rapidly and as thoroughly as possible washed free from adherent matter with normal saline or water, but the washing must not be too long continued. As the extract is afterwards sterilized by boiling there is no fear of infection from any slight trace of adherent material. The strip of intestine is then laid with the mucous surface upward upon a plate of clean glass, and the mucous membrane scraped off with a broad blunt knife, the blade being held perpendicularly to the surface, and five or six inches being cleared at a time. The scrapings are next passed through a fine sausage machine or disintegrator, from which they come out as a homogeneous, soft, semi-fluid mass. This mass is thoroughly mixed up for about five minutes in a mortar, with an equal volume of a dilute solution of hydrochloric acid, containing about 0·4 per cent. of hydrochloric acid, made by adding 10 c.c. of pure, strong hydrochloric acid to a litre of water. The mixture is then placed in a beaker, and, while being stirred, is raised to the boiling point. Finally, sodic hydrate is added until the mixture just remains acid to litmus paper. The resulting preparation is given by the mouth, either as it is, or after removal of the coarse, precipitated proteid. The solution if kept in a bottle roughly sterilized by boiling water will, if left slightly acid, keep well for three or four days. It should not be used later than this because it slowly loses activity, which takes place more rapidly in alkaline solution, for which reason, also, it is best to keep in acid solution.

#### DESCRIPTION OF CASES

Case I. A man of 25 years, by occupation a street car conductor, or driver, was admitted to the Royal Infirmary, Liverpool, under the care of one of us (J. H. A.), on Sept. 14, 1904, suffering from polyuria, loss of weight, weakness, and excessive thirst.

The urine on admission measured 3400 to 3700 c.c. (110 to 120 ozs.), and contained on an average 195 grams (or 3000 grains)

of sugar. Placing the patient on a diabetic diet did not materially reduce the output of sugar. On Sept. 24 the patient was put upon 1 drachm (3.6 grams) of sodium bicarbonate daily, and this was maintained until Nov. 18. On Oct. 11 the patient was placed upon codeia,  $\frac{1}{2}$  grain (0.03 gram), three times daily, which was maintained until 24th Nov. No decrease of sugar followed the administration of the codeia, the amount being maintained unaltered till 23rd Nov., when the patient was put on phenazonum, 5 grs. (0.3 gm.), three times daily.

After the commencement of the phenazonum the amount of sugar slowly decreased in a fluctuating fashion until at the middle of January, 1905, it lay between 40 grams (600 grains), and 65 grams (1000 grains) daily, and was constant at this level.

Without taking off the phenazonum, the patient was given by the mouth extract of duodenal mucous membrane prepared as above described; half an ounce (15 c.c.) three times daily, and on Feb. 8, 1905, the amount given was doubled.

The amount of sugar during the first three weeks after the treatment began showed no alteration, but on the 28th Feb., 1905, there occurred a sudden drop to 32 grams (490 grains). On March 2 the amount of sugar was 25 grams (390 grains), and for some time after this the amount fluctuated between 21 grams (300 grains) and 30 grams (450 grains) daily. The amount of sugar continued to drop, and about the end of May the urine became entirely free from sugar, the patient increased in weight, and the polyuria disappeared. The patient returned to his work, and shortly afterwards stopped coming for the extract. The patient remained well, and continued at his work, as an electric street car driver, until the 14th Aug., 1905. About this time he contracted a cold as the result of a chill obtained at his work, and from this date he grew weaker and lost in weight. On Oct. 13 the patient returned to hospital, he had about 4 to 5 per cent. of sugar on a daily quantity of 2500 to 3000 c.c. (80 to 100 ozs.) of urine. In spite of treatment with the extract there was no material reduction in the sugar, and it was soon discovered that the patient

was suffering from phthisis, which progressed rapidly, and on Dec. 17, 1905, the patient died.

The *post-mortem* examination showed that the pancreas was to all appearance normal, the duodenum had undergone too much alteration for histological examination, and the immediate cause of death was caseous pneumonia of the lungs due to tuberculosis.

Although this first case terminated fatally it is to be observed that for a considerable period after treatment with the extract, the urine was entirely free from sugar. It was this positive result, apparently following the administration of the extract, which led us to continue the treatment in the other two cases which we have to record.

Case II. The case was that of a boy *aged 7 years*, seen by one of us (J. H. A.) in consultation with Drs. Macfie Campbell and Graham Martin.

The patient was never robust in constitution, but had had no very serious illness. He was fairly well during the early part of the year 1905; his mother, who had been absent from home in June, noticed on her return that he had lost flesh, was much paler, languid, always thirsty, and had a huge appetite.

On July 3rd, the patient was seen by Dr. Martin, who found that the urine had a sp. gr. of 1040, and contained 8.3 per cent. of sugar on a daily quantity of 2170 c.c. (70 oz.), that is 179 grams of sugar daily in a child aged 7 years.

The patient was immediately put on a strict diabetic diet, except a small amount of torrified toast, which was also stopped on 13th July, and gluten bread substituted.

He was given phenazonum in 5 grain doses three times daily, and also acid extract of duodenum, prepared as above described in, at first, 2 drachm doses (about 8 grams) thrice daily, and in a week this was doubled in quantity. From the 14th July till the 18th of August, the patient, on account of constipation, was also given *sodii sulphas effervesces*.

The progress of the case is shown in the following table :—

Date 1905	Daily amount of urine in c.c.	Percentage of sugar	Total daily amount of sugar in grams	Weight of patient in kilograms
July 3	2170	8.3	179	18.659
„ 5	The patient was dieted and also given acid extract of duodenal mucous membrane			
„ 7	1980	4.3	85	18.955
„ 9	1890	5.1	96	18.909
„ 12	1860	4.0	74	18.773
„ 14	1610	3.2	52	19.114
„ 17	2170	2.6	56	19.409
„ 19	2170	3.9	85	19.432
„ 21	2360	3.2	76	19.750
„ 23	2360	2.3	54	19.773
„ 24	1460	2.4	35	—
„ 26	2360	0.625	14.7	19.795
„ 28	2570	0.812	20.9	19.682
„ 30	2170	1.083	23.5	20.090
„ 31	2050	1.063	21.8	20.090
Aug. 2	1920	0.771	14.8	20.205
„ 4	1920	0.521	10.0	20.455
„ 6	1800	0.583	10.5	20.455
„ 10	2040	0.417	8.5	20.500
„ 15	1950	0.250	4.9	20.545
„ 18	2420	0.125	3.0	20.295
„ 20	1420	Trace	—	19.864
„ 24	1300	„	—	19.886
„ 30	1180	Absent	—	19.773
Sep. 5	1270	„	—	20.090
„ 13	1460	„	—	20.568
„ 19	1610	„	—	20.590
„ 25	1270	„	—	20.590
Oct. 1	1490	„	—	20.590
„ 11	1360	„	—	20.545
Nov. 20	—	„	—	20.654
Dec. 26	—	„	—	20.932

NOTES.—The initial drop down to 74 grams on July 12, may probably be ascribed to the dieting. On July 13, the small amount of dried toast was discontinued which caused a further small decrease.

A marked drop occurs on July 24, and is continued on the following days. By August 18, the percentage of sugar has fallen to the limit of error of the method used (Gerrard's modification of Fehling's method). Shortly after this the urine showed no trace of reduction on qualitative testing by ordinary Fehling's solution. The phenazonum treatment was reduced to one dose daily on October 27, and stopped entirely on November 14. The duodenal extract was reduced to one dose daily on November 20th, and discontinued completely on December 2nd. Up to the time of writing the urine remains entirely free from sugar.

*Case III.* A girl, aged 9 years, a patient of Dr. Matthews, of Blundellsands.

It is difficult to judge how long the child had suffered from glycosuria, before it was discovered (November 12, 1905). For three months or so it was noticed that she was getting thin, but as she was growing taller, and was active and strong, no special attention was paid to the thinness. It is known, however, that she weighed 68 pounds (30.91 kilograms) a year ago.

The first sample of urine examined, was passed on November 12, 1905, at 1 p.m., the sp. gr. was 1040, and the sugar estimated volumetrically by Fehling's method was 10 per cent.

The child was at once put on a stricter diet, but carbohydrate was never completely excluded throughout the entire case, potato being allowed at dinner time, and a small quantity of milk. Manhu bread, which contains some starch, was given up to Nov. 19, after which diabetic bread (Callard and Co.) was employed. Phenazonum and alkaline carbonates were administered during the first ten days, but later were entirely discontinued, and the case was treated, from the commencement of the employment of the acid extract of duodenum, on that alone.

Throughout the case the urine was only collected from 8 a.m. to 8 p.m., so that for purposes of comparison for twenty-four hours the figures ought to be doubled, but otherwise they are strictly comparable throughout.

The following table shows the progress of the case :—

Date 1905	Amount of urine in c.c. from 8 p.m. till 8 a.m. daily	Percentage of sugar	Total sugar in this period in grams	Weight of patient in kilograms
Nov. 13	740	8.0	59.2	29.090
„ 15	740	6.0	44.4	—
„ 17	740	6.5	48.1	29.090
„ 19	740	5.5	40.7	29.140
„ 21	740	3.0	22.2	—
„ 23	560	3.0	16.8	29.090
„ 25	775	3.0	23.2	—
Acid extract of duodenal mucous membrane given after this time.				
„ 27	530	2.75	14.5	29.540
„ 29	620	2.25	13.9	—
Dec. 1	530	1.5	7.9	—
„ 3	340	1.0	3.4	—
„ 5	590	1.5	8.8	30.000
„ 7	510	1.0	5.1	—
„ 9	370	0.5	1.8	—
„ 11	590	0.75	8.8	—
„ 13	370	0.75	2.7	—
„ 15	500	0.50	2.5	—
„ 17	460	0.30	1.4	—
„ 19	460	0.20	0.9	—
„ 21	Diarrhoea	—	—	—
„ 23	220	Absent	—	29.540
„ 25	470	„	—	—

NOTES.—The initial drop between November 12 and 15 probably arose from the stricter dieting, also that between November 19 and 21 arose from stoppage of the Manhu bread, and substitution of starch-free bread. The amount then remained stationary for a week until the acid extract of duodenal mucous membrane was given, when, without further change in the diet, and without complete stoppage of carbohydrate diet, the sugar fell ultimately to zero. A decrease is also seen in the amount of urine secreted.

During the treatment of the first case, two other patients in Dr. Abram's wards were also treated, the results were negative, but the periods of observation were short, and the dose administered was not adequate in amount, so that no conclusion can be drawn from these cases.

## CONCLUSIONS

No sweeping conclusions can be drawn from such a small number of cases, and they are here given as preliminary, and in order to excite further work upon the subject.

Still, the prognosis in diabetes occurring in young patients is regarded as so unfavourable, that it is very remarkable that in two cases, such as Nos. II and III, where the amount of sugar was so high, complete absence of sugar should be attained so rapidly following the use of the extract.

As to the cause of the glycosuria and the recovery from it in these cases, if a causal connection exists, it is most probable that the cause of the glycosuria is a failure of the chemical excitant from the duodenum, and that this in the end would lead to permanent abolition of the internal secretion of the pancreas. The supply in the extract of the stimulant to the pancreas restarts the internal secretion, and since the glycosuria does not reappear on stoppage of the extract, the administration appears to stimulate the functional activity of the duodenum. The cases, however, still require further watching, and the number of cases requires to be increased before definite conclusions can be drawn.

## THE PHYSIOLOGICAL PROPERTIES OF 'WEST AFRICAN BOXWOOD.'

By R. J. HARVEY GIBSON, M.A., F.L.S., *Professor of Botany in the University of Liverpool.*

(Received January 11th, 1906)

In the summer of the present year I was asked by the Amalgamated Society of Shuttlemakers to give an opinion on the nature and properties of a timber used in the manufacture of shuttles in Lancashire and Yorkshire, and believed by the workmen to have an injurious effect on the health of those employed in the trade.

On enquiry I found that the wood was known under the name of 'West African Boxwood,' and that it had been in use during the past few years for shuttle manufacture, though, at first, only sparingly and intermittently. The recent increase in its use was due to its cheapness as compared with Persian Boxwood and to its freedom from knots, permitting thereby a larger number of blocks being obtained from the log.

I was also informed that during the past two or three years there had been many cases of illness amongst the workmen handling the wood in the factories, and that an impression had got abroad that the sickness was due to some poison given off by the wood during the process of manufacture of the shuttles. The symptoms complained of were headache, sleepiness, running at the nose and eyes, chronic sneezing, giddiness, faintness and weakness, loss of appetite, shortness of breath, nausea, etc. The patients exhibited as well a pale yellowish or greenish colour on the face and body, accompanied by a peculiar 'camphor' or 'Turkey rhubarb' odour from the breath and skin. After weeks of intermittent illness these symptoms had, in two or three cases, culminated in pathological conditions which had resulted in death, the death certificates registering 'cardiac asthma,' or 'cardiac incompetence.' I was also informed that the men who were affected had, in the course of their work, to stop, hold on to some support and gasp for breath. Samples of the wood, together with a quantity of the

sawdust and shavings, were sent me for investigation by Mr. Isherwood, Secretary of the Shuttlemakers' Society. The enquiry, obviously, resolved itself into (a) the discovery of the botanical name of the wood and its economic history ; (b) its chemical characters ; (c) the physiological properties of any extract which might be obtained from it. I should like at once to acknowledge the very great assistance I have received from my colleague, Dr. A. W. Titherley, Lecturer on Organic Chemistry, and from Miss Sowton and Professor Sherrington, F.R.S., in the purely chemical and physiological aspects of the investigation respectively. Without their aid the work could not have been accomplished. I am also indebted for information on several points to Messrs. Joseph Gardner and Sons, of Bootle ; to Dr. Legge, Chief Medical Inspector to the Factory Department of the Home Office ; to Lord Mountmorres, Director of the Liverpool School of Commercial Research in the Tropics ; and to B. Daydon Jackson, Sec. L. Soc.

It was obviously of primary importance to determine what was the botanical source of the wood. As all who deal in timbers are aware, it is often extremely difficult to collate a popular name with a botanical source, and the present instance proved to be no exception to the rule. A glance at the microscopic characters of the wood at once revealed the fact that, though called 'boxwood,' it was not a species of *Buxus* at all events. The wood has been apparently confounded with West Indian Boxwood ; but that wood is, according to Stone (*Timbers of Commerce*, p. 169) a member of the Bignoniaceae, 'reputed to be *Tabebuia pentaphylla*, Hemsl,' having histological characters resembling those of *Buxus*. The wood supplied to me was certainly not 'West Indian Box,' and from its port of shipment, one was led to look for another botanical origin. I was finally led to believe it to be *Sarcocephalus Diderrichii*, De Wild. and Dur. (in De Wildeman, *Notices sur les plantes utiles ou intéressantes de la flore du Congo*, Brussels, 1903), a member of the order Rubiaceae. According to the 'Guide de la Section de l'État Indépendant du Congo à l'Exposition de Bruxelles-Tervueren', 1897, the wood was known also as 'yellow grey box' (Bois-gris-jaunâtre), and said to be shipped from Mayumbe, W. Africa. I found that it

was also known as 'Yellow Congo Mahogany' (acajou jaune du Congo). It was said to be characterised by the presence in it of small pockets containing a solid matter of yellow ochre colour and small, quite translucent, prismatic crystals. It was called by the natives 'N'gulu Maza' or 'N'gulu na mai' ('river' or 'water hog'), from the supposed resemblance of its rough grained bark to the skin of the wild boar or hog. The wood is of a pale golden colour when freshly cut, but rapidly fades to a yellowish-white, with brightly-coloured patches. It forms a capital cabinet wood, difficult to work on account of its cross grain, but still capable of delicate manipulation owing to its hardness, and taking on a high polish. The specimens which were supplied to me agree in all respects with this diagnosis.

The wood has also been confounded with 'East London Boxwood' (*Gonioma Kamassi*, E. Mey.), a member of the Apocynaceae, also a poisonous order, although I have not tested that wood chemically or physiologically. In general appearance the wood of this species closely resembles that of *Sarcocapalus*, but the tapering medullary rays of the former would seem to distinguish it under the microscope from West African Boxwood. Personally, I do not think very great stress can be laid on minute histological characters, for the size of the vessels, thickness of the medullary rays, and other characteristics must vary greatly according to the climatic conditions under which the tree has grown. Undoubtedly such histological characters are not to be neglected, for it is well known that the woods of different species from the same order show family resemblances; but I do not believe that it is possible with absolute certainty to refer a timber to its species from histological features only. *So far at least, as I can judge*, the timber known as 'West African Boxwood' is not identical either with 'South African Boxwood' or with 'West Indian Boxwood.' South African boxwood is shipped from Mossel Bay and Knysna, and is an inhabitant of South Africa, while the subject of the present investigation is a native of the Congo basin and the Cameroons; in any case, the wood, whatever be its source, possesses the chemical and physiological characters described below—the point of importance at present.

After a consideration of the circumstances governing the mode of manufacture of the shuttles it seemed to me that the injurious symptoms might be accounted for by the absorption of some poisonous alkaloid present in the fine dust produced during the manufacture of the shuttles, and this idea was strengthened by the fact that the plant appeared to belong to an order well known to possess alkaloids, viz., the Rubiaceae. On enquiry I found that the logs were first of all sawn into lengths from which blocks of the proper size (12 in.  $\times$  1 $\frac{1}{2}$  in.  $\times$  1 $\frac{1}{2}$  in.) were cut. The edges of the block were then 'dressed,' and the block 'bored' and 'tipped.' The interior was afterwards cut out and the ends turned in a lathe, the shuttle being finally 'finished,' so far as the wood was concerned, by being polished with sand paper. The whole series of processes, save the 'finishing,' is carried out by machinery. Finishing is, however, done by hand, and each finisher works, I was told, for about one hour per diem at this phase of the manufacture. Naturally much dust is created, more especially in the final sand-papering stage, when the workmen are extremely lightly clad, being often naked from the waist upwards. I was told that the sickness was most prevalent among the workmen whose duty it was to saw the logs into 'blocks,' and among those who polished the chiselled shuttle with sand paper into the final finished product. Such men were constantly enveloped in a cloud of fine dust, 'like smoke,' given off by the saws or lathes, and were under circumstances such that the inhalation of the dust or absorption of it by the skin was unavoidable.

The first thing to be done was, obviously, to extract the alkaloid from the dust—assuming that an alkaloid was present. This was accomplished in the following manner:—A kilogram of the dust was extracted with four litres of water, at a temperature of 70-80° C. To this was added 100 c.c. of concentrated hydrochloric acid, the whole being agitated and digested for four hours. The liquor was then filtered, and the ligneous material well washed. The combined filtrate and washings were concentrated down to two litres, such resinous matter as was present, and which had separated out was filtered off and the clear brown solution, was precipitated by an excess

or potassium-bismuth iodide. A voluminous, orange-brown precipitate was obtained (a double compound, alkaloid-bismuth-iodide) which was collected and well washed with water. The precipitate was then decomposed by an excess of a 10 per cent. solution of caustic soda, in order to liberate the free alkaloid, and the mixture was extracted twice with about 100 c.c. of freshly-distilled chloroform. A certain amount of emulsification was unavoidable. After settling, the chloroform extract was washed with water and, finally, all traces of moisture were removed by filtration. The chloroform solution, which was deep brown in colour, was then distilled on a water bath to remove most of the chloroform, the last traces being removed by heating at 80-90° C. in an open glass dish. A thick, dark brown syrup remained over, consisting of the free alkaloid which, on cooling, solidified into an amorphous, brittle, glassy solid. It weighed 0.7 gr., representing 0.07 per cent. of alkaloid in the wood. Assuming that it had been possible to extract all the alkaloid, the wood may be taken as containing 0.1 per cent. of the alkaloid.

The alkaloid was then converted into the hydrochloride by digestion with dilute hydrochloric acid (5 per cent.) the whole being evaporated to dryness on the water bath. The residue after evaporation was then dissolved in water and filtered from about 0.2 gr. of resinous insoluble impurity, and the filtrate was diluted so as to contain 0.7 per cent. alkaloid hydrochloride. The alkaloid when examined in detail was found to be very soluble in chloroform and in alcohol (though less so), sparingly soluble in water, but the hydrochloride was readily soluble in water and salt solution. On heating, it decomposes, chars and gives off a vapour with a peculiar and penetrating odour. On adding caustic soda or  $\text{Na}_2\text{CO}_3$ , to a solution of the hydrochloride the free alkaloid is thrown down as a yellowish, amorphous, curdy precipitate. It was found to be impossible to obtain it in the crystalline form by dissolving it in hot water and cooling. The alkaloid gave the following reactions :—

- (a) Bismuth iodide in potassium iodide gave a bright orange precipitate even in very dilute solutions.
- (b) Iodine in potassium iodide gave a dirty brown precipitate coagulating in dark brown masses.

- (c) Nessler's solution gave a whitish-yellow precipitate.
- (d) Tannic acid gave a gelatinous white precipitate.
- (e) Picric acid gave a yellowish precipitate.
- (f) Platinic chloride gave a yellow precipitate in strong solutions.
- (g) Phosphomolybdic acid gave a thick pale yellow precipitate.

The solutions used for experimental purposes were (a) a solution of the alkaloid in alcohol, and (b) a solution of the hydrochloride in water.

The fluid (a) actually used was a 1 per cent. solution of the alkaloid in absolute alcohol, diluted to ten times its volume with Ringer's solution. This will be referred to in the experiments to be described, as 'A' solution; the other (b) was a 0.7 per cent. solution of the hydrochloride in water and will be styled 'B' solution.

Before describing the experiments themselves it may be advisable, perhaps, to summarise briefly some of the data furnished me by the Secretary of the Shuttlemakers' Society, amplified by notes kindly put at my disposal by Dr. Legge, H.M. Chief Medical Inspector of Factories, whose attention was called to the matter in consequence of my report on the wood to the Shuttlemakers' Society.

Dr. Legge\* informs me that he examined about 65 per cent. of the men engaged in working the wood in a number of factories visited and found that about 32 per cent. complained of headache lasting for from two to four hours after beginning to work the wood, and continuing for from two to three days after discontinuance. The headaches were described as 'over the eyes or at the temples.' Eleven per cent. complained of sleepiness, the feeling being described by the men as 'maziness' or 'dosiness,' and in some cases being so pronounced as to necessitate a strong effort of will to avoid falling off the bench. Fourteen per cent. complained of running at the eyes, and about 29 per cent. of running at the nose. These symptoms appeared only when Africcan Box was used. (I may add here that other woods are employed for the manufacture of shuttles, e.g., Persian Box, Persimmon, Cornel, etc., as well

\* Since this paper was written Dr. Legge has examined several other factories, and he informs me that the cases he enquired into quite bear out his former investigations.

as W. Indian and S. African Box, above referred to.) The 'running' was not accompanied by any ulceration but local inflammation of the mucous membrane was observable. Breathing was affected in 35 per cent. of the cases noted. The condition was such as to compel several men to give up work on the wood and the poison seemed to affect expiration rather than inspiration, hence leading to the accumulation of mucus in the trachea. About 10 per cent. complained of nausea and faintness. In most cases, two or more symptoms were complained of by the same individual. Thus one man stated that he had suffered from frontal headache, running at the nose, pains in the limbs and shortness of breath. These symptoms disappeared when he changed from the wood to the metal department. Another, a sawyer, complained of frontal headache, dizziness, nausea and difficulty in breathing. This workman left off work for six weeks and recovered, but had a renewal of the attacks a few days after starting work once more. Another 'finisher' suffered from asthmatic symptoms which were, according to his medical adviser, affecting the heart to such a degree that he was recommended three months' rest. After an interval of one month, the workman in question returned to work, but in a few days the same symptoms reappeared. After an interval of two months he once more returned to work and soon began to suffer from dyspnoea, palpitation, and a sensation of suffocation. He has done no work during the last few months and has had no recurrence of the attacks. On the other hand, Dr. Legge informs me that one man who had sawn over one hundred tons of West African Boxwood had suffered no inconvenience at all, and suggests that individual idiosyncrasy may play some part in determining the occurrence or the severity of the symptoms. This, so far as I am aware, agrees entirely with the effect of the poisons occurring in such plants as *Rhus Toxicodendron*, *Primula obconica*, *Asparagus officinalis*, etc. In some cases these plants produce severe eruptions on the face and hands of those touching them, whilst in other cases the poison produces no effect whatsoever. It is possible, however, that the workman referred to may have mistaken the wood on which he was employed for West African Box.

I append here a detailed report on a 'case' kindly put at my disposal by Dr. Hamilton Stewart, of Bradford, as an instance of the specific effect of the dust of West African Box.

*Case.* Aged 38. Employed for between twenty-four and twenty-five years as a 'finisher off' at the shuttle shop in Thornton.

*Previous history and health :—*

- (1) He is a married man with a family of six—all healthy.
- (2) His previous health was good until seven or eight years ago, when the present symptoms began.
- (3) His family history is good. Both his mother and father are alive—the latter has occasional epileptic attacks.
- (4) *Present illness* :—He dates the beginning of the present illness from seven years back. West African Boxwood has only been worked, so far as he remembers, in this shuttle shop for the last ten years, and that only occasionally. It has never, since they began using it, been worked continuously, but only occasionally for varying periods of time. At first he worked the wood a good deal—'dressing up,' and he did not experience any evil effects for about three years. Then the symptoms from which he now suffers, when working with that particular wood, began.

*History of the attack :—*

The attack began with running of the eyes and nose, and a feeling of suffocation. He had a feeling of nausea and sickness, with violent retching. He felt *faint* and *done*, so much so that he felt a desire to lie down—a desire he could not gratify owing to the difficulty he had in breathing. He complained of a slight pain and uneasiness over the cardiac region, and broke into a cold, clammy sweat. His breathing gradually became worse, more rapid and difficult, so that he could neither speak nor swallow. When in the fresh air his symptoms seemed to him to be at first aggravated, and then gradually to pass off.

The duration of the attack depended on the length of time he had stayed at work. If he stayed at work now for, say, ten minutes when this wood was being used, he took two or three hours to recover, but if he remained at work one or two hours, two or three days passed before recovery was complete. Lately it has begun to affect him more quickly than it used to do. If the wood were being used now and he went to work on it, he would be retching violently in about ten minutes. At times he has been working in one of the upper rooms of the workshop, where he could not see and did not know what wood was being sawn up. So soon as the dust began to work its way up, his symptoms began. After one very severe attack he was three weeks at home, and three weeks at Ilkley Convalescent Home to recruit, and finally felt quite well. He returned to his work but, unfortunately, they were using this wood, and that same day he was as bad as ever.

Now that this wood has not been in use at this workshop for a long time, he is quite healthy, strong and active, and has not lost any time.

*Physical Condition during the Attack :—*

He was seated upright on a chair, gasping for breath, with the door of his house wide open. He had a somewhat livid appearance, and the skin was cold to the touch, and covered with a cold, clammy perspiration. His temperature was normal. He could not lie down, and the difficulty of breathing was such that he could just jerk out a word or two in answer to questions. His eyes were red and somewhat swollen, and both eyes and nose were running. There was no ulcerative condition of the inside of his nose, but the mucous membrane was swollen, and he complained of his nose feeling twice the size it ought to do. He complained of no headache or feeling of sleepiness. He kept constantly retching and trying to vomit.

*Circulatory System :—*

He complained of slight cardiac pain and uneasiness, and dyspnoea was marked. The pulse was frequent and about 100 per minute. It was regular but soft, small and easily compressed. The apex of the heart was in its normal position, and the palpation could scarcely be felt.

On auscultation the valve sounds were normal in character, but weak and muffled, and, altogether, gave one the impression that the heart was rapidly failing.

*Respiratory System :—*

Respiration was quick and laboured. The dyspnoea was marked and was of the expiratory kind. He had a great deal of difficulty, evidently, in emptying his chest, and had to sit up straight in his chair and use his forced muscles of respiration.

There was no dulness on percussion or increased resonance.

On auscultation the inspiratory sounds were fairly normal, but expiration was prolonged and piping. Beyond the piping expiratory sounds there were no rhonchi to be heard.

*Digestive System :—*

This seemed normal, with the exception that he complained of a bitter taste in his mouth, and of the violent retching. All the other organs were normal and there was no albumen in the urine.

*Diagnosis :—*

When I first saw him during a severe attack, I was quite at a loss to account for his symptoms. The attack looked somewhat like an ordinary attack of spasmodic asthma caused probably by the inhalation of dust, yet I had never before seen an attack of asthma cause so much cardiac depression in such a short time. On the other hand, it did not quite conform to the ordinary type of cardiac dyspnoea—there was more difficulty with the expiration than is usual with such attacks, and there was no evident cardiac valvular lesion. The attack seemed to come between an ordinary attack of asthma and one of cardiac asthma.

I saw him in several other attacks, and in talking the matter over with him he told me that he had noticed that he always had an attack when they were working this wood. I came, therefore, to the conclusion that it was the dust of this special

wood that was so deleterious to him, and I accordingly advised him to leave his work when they began working West African Boxwood. He tried a respirator with cotton wool in it for a time but he felt suffocated in it and had to discard it.

We may now turn to the discussion of the physiological effect of the alkaloid on the mammalian heart as evidenced by the sphygmographic records kindly prepared for me by Miss Sowton in the Thompson Yates Physiological Laboratories of the University.

The heart used was that of the cat, removed from a recently killed animal, and perfused with a salt solution prepared according to Ringer's formula at body temperature. The flow through the heart was maintained by means of oxygen pressure.

#### EXPERIMENT I (with solution 'A')

(a) *Control Experiments*.—In using solution 'A' it was manifestly necessary to know what share in the results obtained was due to the presence of the alcohol in which the alkaloid was dissolved, so that control experiments were first of all carried out before any of the alkaloid was injected. Three such controls were recorded, and it was found that (using 7 c.c. of alcohol) at the first and second injections the heart beat was almost instantly stopped but for a short time only; at the third injection the heart had become accustomed to the dose, and the effect was masked.

(b) *Observation I* (Fig. 1).—2 c.c. of solution 'A' was next injected by means of a hypodermic syringe into the perfusion tube, about three feet away from the heart, after it had regained its normal beat. The solution 'A' was thus well mixed with the perfusion fluid. The immediate effect was a slowing of the heart and a rapid reduction to zero. The time of injection lasted fifty-five seconds. Ten seconds after the injection was begun the slowing of the heart-beat followed, and twenty seconds later had entirely ceased. The heart remained quiescent for about 180 seconds, or about  $2\frac{1}{2}$  minutes after the injection had ceased. The heart then commenced beating very feebly, and with a much slower rhythm, gradually increasing in vigour and still more gradually in speed.

*Observation 2.*—When the heart had again regained a normal but slower beat, 5 c.c. of solution 'A' were injected in the same way as before, the time of injection lasting rather over one minute (70 seconds). Within half a minute of the first injection the heart began to slow and decrease in vigour, and finally at the end of the injection period came to a standstill. The cessation was permanent for about seven minutes, when the heart began to show feeble attempts at contraction, and a minute later more or less regular but very slow beats (about one beat every five seconds). The heart showed no signs of permanent recovery.

### EXPERIMENT II (with solution 'B')

*Observation 1.*—Solution 'B' was diluted to one in ten with Ringer's saline and 8 c.c. of the mixture was slowly injected (the period of injection lasting 2 minutes, 45 seconds). A few seconds after injection commenced the vigour of the beat rapidly fell to one-sixth of the normal, and in about half a minute fell to nil, the heart remaining quiescent for half a minute after the injection had ceased. It then began slowly to recover, the beats being at first very feeble and slow, but increasing both in strength and in rhythm, until, about three minutes after, the beat was again fairly normal though not quite so rapid as at first.

*Observations 2 and 3.*—Short injections of 10 c.c. of the fluid escaping from the heart produced only a slight effect.

(b) *Control experiment 4.*—At this stage 10 c.c. of Ringer's Saline without alkaloid were injected but no effect was produced.

(c) *Observation 5* (Fig. 2).—The solution 'B' was again used but this time diluted with Ringer's saline to one part in five of the saline. Of this fluid 5 c.c. were injected. The beat rapidly slowed down and ceased twenty-five seconds after the injection had been begun. The injection lasted two minutes, during which time and for sixty seconds longer the heart was entirely quiescent. Then followed a slow and gradual recovery, the rhythm, however, never reaching the speed of the original heart beat. (It is of interest to note in this relation that several of the workmen suffering from the action of the wood-dust

also shewed a very slow pulse, one case, as Dr. Legge informs me, being as low as fifty).

*Observation 6* (Fig. 3).—In this experiment 2 c.c. only were injected but the delivery was very rapid, viz., under ten seconds. The effect was almost instantaneous, the heart ceasing to beat within about five seconds, and remaining practically quiescent for  $1\frac{1}{2}$  minutes, when very slow and feeble pulsations recommenced, gradually, but very gradually, increasing in vigour and rapidity to the normal once more.

All the observations hitherto recorded were carried out by the syringe method of delivery in order to economise the material, which, as will have been seen from the description of the method of preparation, was obtained only by a long and laborious process. The syringe method permits of only approximate estimation of dosage and hence, in the experiments yet to be described, the more exact method of administering the dose in full stream from a flask, containing a mixture of solution 'B' and Ringer's saline made up before-hand to the required strength, was adopted. The flask used was similar to that containing the normal Ringer's saline and was under the same oxygen pressure, and the change from normal saline to drugged saline and back again was effected by means of a system of three-way taps, connecting either flask with the delivery tube immediately before the delivery tube enters the heart.

### EXPERIMENT III (with solution 'B')

*Observation 1* (Fig. 4).—In this experiment carried out as above described, solution 'B' was diluted to 0.023 per cent., and administered for fifty seconds. In all, 33 c.c. of the fluid passed through the heart. The effect was a rapid slowing and final stoppage within thirty seconds of the beginning of the injection; quiescence continued for about a minute after injection had ceased, when the heart began to slowly recover both in vigour and in rhythm.

*Observation 2*.—The same degree of dilution of solution 'B' was employed, but the fluid was administered for six minutes, five seconds, 142 c.c., in all passing through the heart. In this case within half-a-minute the heart had ceased to beat, continued quiescent during

the entire period of injection, and for several minutes afterwards, and, though recovering very feebly and slowly, it appeared to be permanently injured.

#### EXPERIMENT IV (with solution 'B')

Solution 'B' was diluted to 0.0077 per cent. and administered for 1 min. 45 sec. The beat rapidly slowed and decreased in vigour, reaching a standstill, with occasional spasmodic flickers, before the end of the injection. The tracing showed that the heart never recovered, and only evinced occasional spasmodic contractions of feeble power and most irregular rhythm. The heart was undoubtedly permanently injured.

#### EXPERIMENT V

Solution 'B' was diluted to 0.0026 per cent., and administered for four minutes, thirty seconds, 48 c.c. of the fluid in all passing through the heart. The beat declined very gradually, disappearing by the end of three minutes. Recovery was very imperfect, and even when they recommenced, the beats were very much slower and most irregular.

#### EXPERIMENT VI

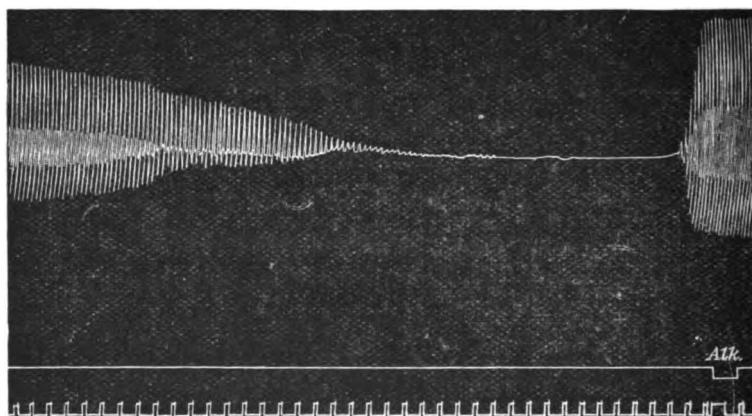
The solution 'B' was still further diluted, viz., to 0.0013 per cent., and administered for about eight minutes, when the solution began to give out. The beat was very slowly retarded, and became gradually feebler; recovery was fair, but never became complete.

#### CONCLUSIONS

The net result of all these experiments is to show that the alkaloid present in 'West African Boxwood' is a cardiac poison, inducing a gradual slowing of the heart-beat and diminution of vigour in the contractile tissue of the heart; that its effect is cumulative, finally producing a cessation of the beat under long exposure to its influence. The fact that the alkaloid is very soluble in saline solution should, it appears to me, be noted along with the fact that the perspiration contains a considerable percentage of salt, and that,

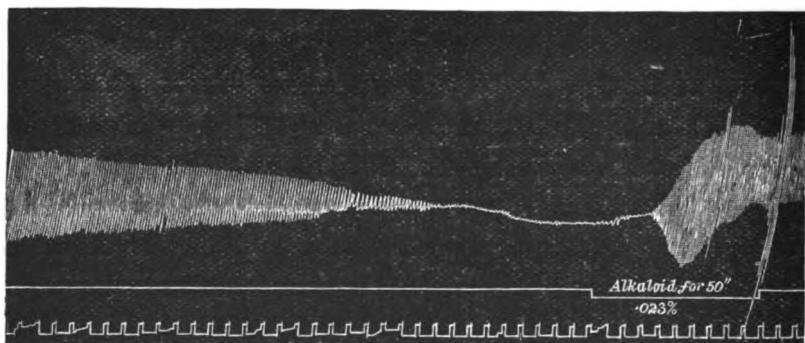
according to Waller (*Human Physiology*, 1896) the palm of the hand (the part of the body most exposed to the action of the dust in the case of the workmen concerned) gives off more moisture, area for area, than any other part of the body surface. I have no doubt in my own mind that the fine particles of ligneous matter of the wood in question falling on the perspiring skin—more especially of the hands—gave up in variable quantity the alkaloid extracted which became absorbed into the circulation, and ultimately affected the heart of the worker and induced indirectly the various symptoms described in the preceding pages.

FIG. 3



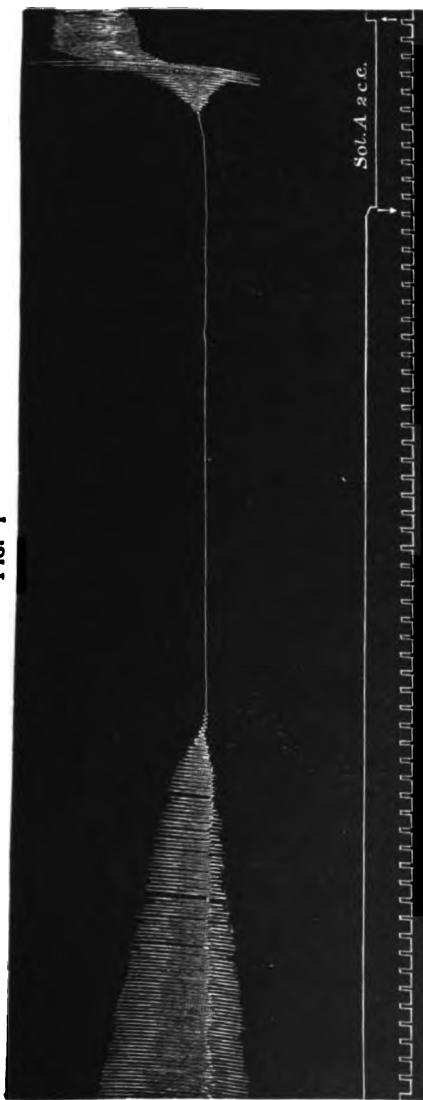
Tracing from cat's heart after injection of 2 c.c. of solution B lasting 10 secs.  
See text, Experiment II, Observation 6. Tracing to be read  
from right to left. Time interval, 5 secs.

FIG. 4



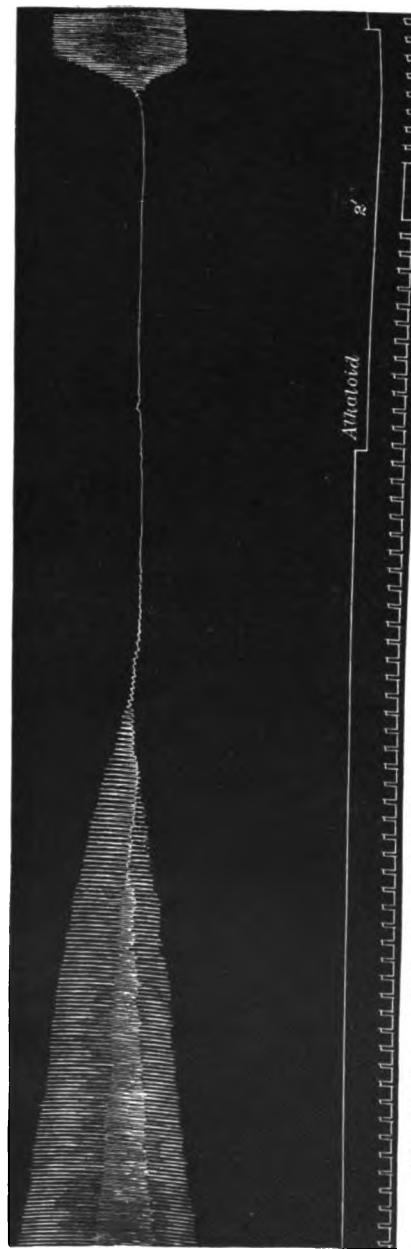
Tracing from cat's heart after injection of 33 c.c. of solution B. See text,  
Experiment III, Observation 1. Tracing to be read from right to left.  
Time intervals, 5 secs.

FIG. 1



Tracing from cat's heart after injection of 2 c.c. of solution A. See text, Experiment I, Observation 1.  
The tracing is to be read from right to left. Time record in 5 sec. intervals.

FIG. 2



Tracing from cat's heart after injection of 5 c.c. of solution B. See text, Experiment II, Observation 5.  
Tracing to be read from right to left. Time intervals, 5 secs.



## FILTRATION AS A POSSIBLE MECHANISM IN THE LIVING ORGANISM

By LEONARD HILL, M.B., F.R.S., *Lecturer on Physiology, London Hospital, Medical School.*

(Received January 16th, 1906)

Since the teaching of Ludwig, filtration has generally been regarded as a mechanism by which might be explained the passage of lymph through the capillary wall, and of dilute urine through the glomerulus. Now it appears to me that no difference of pressure on either side of a membrane, such as might produce filtration, can exist in the body, owing to the structure of the tissues and the physical conditions under which the circulation proceeds. The contents of the living cells are a colloidal solution, about 80 per cent. of which consists of water, and this solution must practically, like water, transmit pressure equally in all directions. The only exception to this will be in the case of structures in which the colloidal matter is in an anisotropic condition, a condition of structure produced by strain in definite directions. The connective tissues are of such a nature, and confine the cells of the body into different organs or parts, e.g., the capsules of glands, and aponeuroses of muscles. In each organ or part we have the cells (muscle fibres, gland cells, 80 per cent. of which is water) surrounded by capillary networks and tissue lymph. An examination of an injected microscopic specimen of kidney, pancreas or muscle at once suggests that pressure transmitted through the wall of the capillaries is transmitted to the immediately surrounding structures equally in all directions.

Let us first consider the glomerulus of the kidney in which filtration is supposed to take place. The pressure in the glomerular capillaries, which is supposed to filter fluid through the capsule, must

be transmitted equally through the practically fluid cellular structures surrounding the glomerulus, and, as shown by Fig. 1, must compress equally the capsules, the exit-tubule A, and the neighbouring convoluted tubule B. It seems most improbable that the capsule should be held open by its own elasticity and act as a filter funnel, for are not the cells of which it is composed formed of a colloidal solution of which about 80 per cent. is probably water? Even if the capsule could act in the way supposed, it seems clear that the exit tube leaving the capsule is exposed to as great a pressure as exists within the capsule, for, firstly, the pressure transmitted through the wall of the glomerular capillaries must reach the exit tubules and, secondly, the

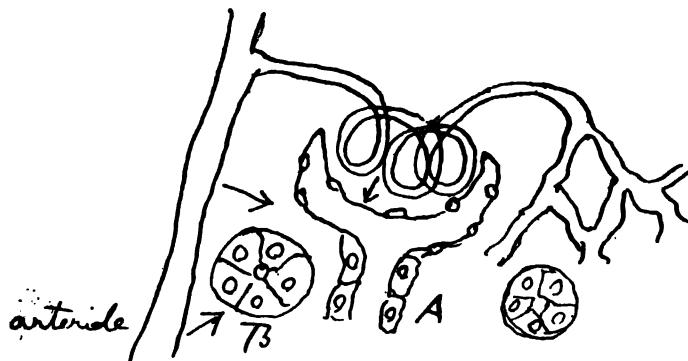


FIG. 1

arterioles which feed the glomeruli run among the convoluted tubules, and the pulsatile force transmitted through the wall of these arterioles cannot be less than that transmitted through the wall of the glomerular capillaries. Further, the loops of Henle and the collecting tubules are surrounded not only by capillaries, wherein we have no reason to suppose the pressure is less than in the glomerular capillaries, but also by the larger branches of the renal artery, through the walls of which the pulsatile force of the heart is transmitted, and this force cannot be less than the pulsatile force which is transmitted through the wall of the capillaries.

The conditions then are shown in Figure 2.

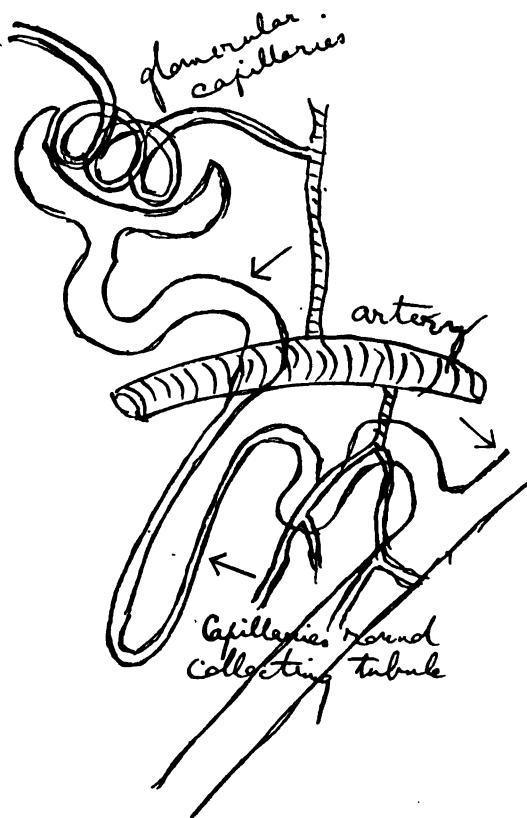


FIG. 2

The collecting tubes are exposed to no less a blood pressure than the capsules or secreting tubes.

Looking at the microscopic structure of the tubules in an unhardened section of kidney, it seems to me impossible that the tubules can stand open by their own elasticity. Their walls are formed of a watery colloidal solution, and practically must transmit the capillary pressure equally in all directions. I think, therefore, no filtration pressure can exist. I believe that the glomerular venule is smaller than the arteriole, not so as to increase the blood pressure in the glomerulus, but because the capsule secretes water, and a smaller volume of more concentrated blood passes away from than enters the

glomerular vessels. The difference between the diameters of the afferent and efferent vessels probably can be taken as a measure of the amount of concentration the blood undergoes.

Holding to the above view of the conditions in the kidney, I do not conceive as possible the distribution of pressure shown by Fig. 3, which is given in Starling's text book (after Morat).

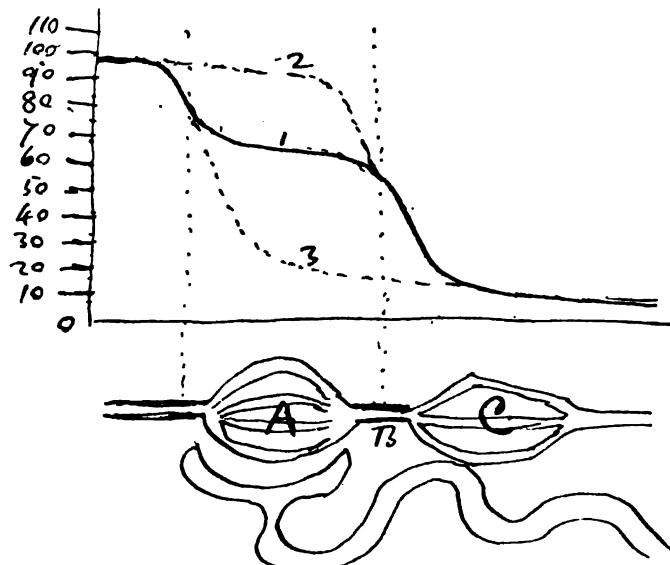


FIG. 3

The curves, 1, 2, and 3, are supposed to show the pressure in the glomerular capillaries, (1) in the normal condition, (2) in the condition of vaso-constriction, (3) of dilatation of the venule B. It seems impossible that there should be the wide difference of pressure between A and C shown in curves 1 and 2, for if such a pressure existed C would be obliterated by the pressure transmitted through the walls of A; also, C would be obliterated by the pressure of the urine supposed to be filtered through the walls of A into the capsule. The obliteration of C would at once lead to the pressure in C approximating to that in A. There is, moreover, no evidence that the venule B is a contractile tube.

I hold the view that the whole kidney is expanded at each stroke of the heart by a force which equals the systolic pressure minus that

part of the pressure which is spent in expanding the walls of the renal vessels. In other words, the whole kidney substance is expanded at uniform pressure which is equal to the pressure in the renal vein. The blood flow is maintained by the fall of pressure gradient between arteries, capillaries, and veins, the pressure which is transmitted through the walls of the arteries and capillaries being less than that transmitted directly through them.

The kidney expands with blood, as a whole, during systole, and shrinks during diastole, and there is nothing in favour of the supposition that the pressure on one part of the renal tubule can be greater than on another. The pressure of the secretion cannot be normally greater than the pressure *in* the veins, for otherwise the secretory pressure would compress the veins. If the secretion be obstructed the pressure of both it and of the blood in the veins must rise together. The secretion moves onward, I take it, by phenomena of adsorption.

The fact that obliteration of the renal vein stops secretion altogether completely agrees with the views put forward by the writer.

I base my opinions on the experimental observations which I have made upon the brain. I found that it takes the same pressure to reduce the expansion of the brain into a trephine hole, as to reduce the amount of blood in the pial capillaries. This pressure is the same as the pressure in the venous sinuses, and the pressure of the cerebro-spinal fluid. The whole brain substance and the cerebro-spinal fluid are at the same pressure, that is the pressure which is transmitted through the walls of the blood vessels—the force of the heart minus that part of the force spent in overcoming the viscosity of the blood and of the vascular wall.

The pressure *within* the capillaries is, of course, higher than in the venous sinuses, and this pressure gradient maintains the flow, but the pressure transmitted through the wall of the capillary is equal to—not greater than—that in the veins. Thus the venous outflow cannot be impeded by the pressure transmitted through the wall of the capillaries. The pressure thus transmitted being equal to the cerebro-spinal fluid pressure, there exists, in my opinion, no force for producing filtration of this fluid.

Let us suppose that the above conclusion is wrong, and that the pressure in the capillaries of the choroidal fringes is greater than in other cerebral capillaries, and that cerebro-spinal fluid is filtered through by this excess of pressure, then this fluid being at a higher pressure would compress the veins and capillaries in the surrounding parts of the brain wherein the pressure is lower, and the flow being obstructed therein, the pressure in these parts would quickly rise to that of the cerebro-spinal fluid. In other words, the filtration would come to an end.

The only condition under which filtration seems possible is when the skull is freely opened so that the cerebro-spinal fluid can escape. In such case we have a capillary pressure (in the horizontal posture) about 110 mm. of water higher than that of the atmosphere. In the vertical head down posture it might be over 100 mm. of mercury.

In a limb enclosed by the skin the conditions are the same as in the brain or kidney. The bony nature of the cranial wall limits the expansion of the brain with blood beyond a certain part, and the connective tissue of the skin and aponeuroses, and of the renal capsule limits expansion in the same way.

In the limb, just as in the brain, the pressure of the blood on the living cells (muscle-fibres) and on the lymphatics is the same, and the conditions do not allow of a filtration pressure which can explain the flow of lymph. Only when the skin is wounded can a filtration pressure come into play.

The flow of lymph is maintained, not by a *vis a tergo*, but by the pulsatile expansion and shrinking of the organs, by the respiratory pump, by the expressive action of the muscles, and by gravity acting in changes of posture.

A simple illustration may make clear my point of view.

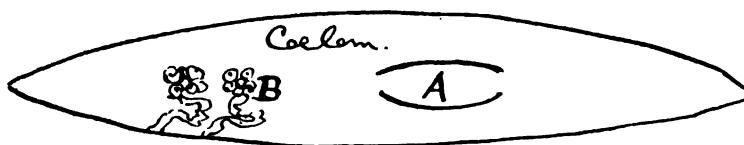


FIG. 4

In the simple type of animal represented in Fig. 4, let A represent a pulsatile heart, which by its contraction stirs up the fluid which fills the coelom. An excreting gland B, is obviously exposed to the same fluid pressure, both in its secreting and conducting part, and filtration can take no share in the process. In the mammal, similarly, the secreting part and the conducting part of a gland or lymphatic are alike surrounded by capillaries, and are equally exposed to the pressure of the blood transmitted through the walls of these vessels.

It is the chemical activity of the living cells, phenomena of adsorption, osmosis, etc., which produce the turgescence of the cells, the secretion of lymph, of urine, and of secretions, and alterations in the circulatory system do not act by mechanically modifying a filtration pressure, but by modifying the activities of the living cells.

Since writing the above, I have read a paper of W. Filehne and Biberfeld, just published in Pflüger's *Archives*. Filehne and Biberfeld express the same view as to the kidney as is set forth in this paper, a view which I have taught for a long time.\* The following passage gives Filehne and Biberfeld's conclusions:—

'Ja selbst wenn ein Filtrationsdruck Wirklich die Bowmanschen Kapseln mit Flüssigkeit füllen könnte, so würde bei der alsdann erfolgenden Fortpflanzung der Drucksteigerung über das Parenchym der ganzen Niere hin, eine Kompression der so sehr langen und so vielfachgewundenen Harn-kanälchen stathaben. Das abgeschiedene Harnwasser würde sich also durch eben den Druck, der seine Filtrirung veranlasst hat, selber den Ausweg verlegen. Ein Abfliessen, ein Fortschieben des Harn-wassers is eben nur möglich, wenn ein "Druck" die Flüssigkeit von hintenher treibt, der den allgemeinen Parenchymdruck nicht steigert. Dies könnten z. B. osmotischen Druckdifferenzen leisten nicht aber ein hydrostatischer Filtrationsdruck.'—*Arch. f. d. ges. Physiol.*, January 4, 1906.

\* See note in *Recent Advances of Physiology*, p. 618, the preface of which is dated November 26, 1905.

## THE PHARMACOLOGICAL ACTION OF DIGITALIS, STROPHANTHUS AND SQUILL ON THE HEART<sup>1</sup>

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It not infrequently happens in the treatment of disease that although certain drugs appear to be indicated, they do not produce their usual effects. In some instances this is due to the poor quality of the preparations prescribed, and, for certain drugs, this contingency cannot be foreseen and provided against at the present time. In many pharmaceutical preparations the active principles have been well and carefully studied, and chemical methods have been devised for estimating them exactly; in others, such as anti-diphtheritic serum, chemical methods are not at present available, and they are therefore standardized physiologically. This method is the only reliable one for the standardization of digitalis, strophanthus, squill, ergot, and Indian hemp.

1. Towards the expenses of this research a grant was made by the British Medical Association, on the recommendation of the Scientific Grants Committee of the Association.

The group of cardiac tonics is characterized by the content of one or more glucosides as the active principle. These glucosides cannot be prepared in a pure state ; first, because their chemistry is so little known, and, secondly, because the different chemical processes suggested for their extraction produce different substances, or at all events substances with very different activities. The quality of the active constituents varies not only in different seasons, and in plants grown in different soils, but also in different parts of the same plant. Moreover, in the attempt at isolation much of their potency is lost, so that these so-called active principles require standardization even more than the galenical preparations. Thus in the Pharmacological Laboratory of Cambridge University several 'digitalins' and 'strophantins' have been shown to be worthless, and one beautifully crystalline 'digitoxin' was quite inert.

It is necessary therefore, before any comparative investigations with these drugs can be attempted, to obtain an indication of the strength of the preparations to be used. With this object in view, I obtained good average samples of the drugs, digitalis, squill, and strophanthus, which were available in March ; and tinctures were prepared from these, exactly according to the directions in the British Pharmacopoeia of 1898. This is certainly a matter of considerable importance, for other tinctures, obtained from equally good specimens of the crude drugs, but less carefully prepared, were decidedly less toxic in their action. I shall in future refer to these galenicals as the standard tinctures.

#### STANDARDIZATION

Experiments were first made with the carefully prepared tinctures in order to ascertain their relative potency and toxicity, and to form a standard with which other tinctures could be compared. These drugs have a specific action on the heart, and the type of action is the same on every vertebrate animal, so that, just as we standardize diphtheria toxin by determining the minimal lethal dose necessary to kill a guinea-pig, so we can standardize the members of the digitalis group

by determining the minimum amount of the drug necessary to kill some animal. Guinea-pigs may be employed for these experiments, but as frogs answer every purpose, I eventually standardized my preparations by determining the smallest dose of the drug which, when injected into the dorsal lymph sac of a frog, caused death by arresting the heart in systolic contraction.

Physiological standardization is, of course, not new ; it has been suggested by Gottlieb<sup>1</sup> that digitalis preparations be standardized ; by Houghton<sup>2</sup> that preparations of ergot be assayed by marking the gangrene after feeding roosters with the drug ; by Barger and Shaw<sup>3</sup> that the activity of the digitalis group be determined for the frog's heart ; and by Dixon<sup>4</sup> and Cushny that digitalis, strophanthus, squill, ergot, and Indian hemp should all be standardized ; and Dixon has suggested methods by which these may be effected. Of course it is not suggested that the dose of any of these drugs necessary to kill a frog bears any direct relation to the dose necessary to produce an effect on man. But supposing that 5 minims and 1 minim are found to be the minimal lethal doses of two tinctures respectively for a frog of 25 grammes weight, then in order to obtain corresponding effects, a man should take as a dose five times as much of the latter as of the former. Dixon and I<sup>5</sup> suggested standards with which to compare commercial tinctures, and as it is these standard tinctures which have been employed in these experiments, I give their relative toxicity.

The minimal lethal doses are as follows :—

Tinct. Digitalis	= 2½ m.	for a 20 gram. frog.
Tinct. Squill	= 2½ m.	„ 20 „
Tinct. Strophanthus	= ¼ m.	„ 17 „

Hence the relative toxicity of the three standard tinctures of digitalis, squill, and strophanthus is such that the first two are about equal in potency, while strophanthus is nearly nine times as toxic as

1. Gottlieb, *Berlin Medical Congress*, XIX, 1901.
2. Houghton, *Therapeutic Gazette*, Vol. XXVIII, p. 450, July, 1898.
3. Barger and Shaw, *The Year-book of Pharmacy*, 1904.
4. Dixon, *Pharmaceutical Journal*, July 29, 1905.
5. Dixon and Haynes, *Medical Magazine*, January, 1906.

either. Fraser<sup>1</sup> finds that the M.L.D.\* for strophanthin per 100 grains of frog or rabbit equals 0.00005 grain, and says that strophanthin is a little more active than the pure alcohol extract of strophanthus seeds. He states that it is 300 times more powerful than Merck's purest digitalin, and 3,000 times more powerful than an English digitalin. As, however, these glucosides are known to vary enormously, this observation is of little value without the M.L.D. of the digitalins being stated.

It may be objected that although the strophanthus is so much more toxic than digitalis when the drugs are injected hypodermically, the results will be different when the drugs are administered by the mouth. Such variation is not unusual: for example, a single dose of lead, if taken by the mouth, is very slightly toxic, because it is not absorbed to any extent; but the same dose injected hypodermically will produce symptoms of poisoning. And, again, arsenic is infinitely more poisonous than iron when the two are administered by the mouth, because the arsenic is absorbed and the iron is not to any extent. But if the two are injected, there is not much difference between their toxic effects. Hence it might be objected that strophanthus given by the mouth is less effective because of deficient absorption. I decided, therefore, to determine how far the comparative toxicity held when the drugs were given by the stomach. For this purpose certain experiments were conducted on mammals.

#### INTRODUCTION OF DRUGS INTO THE STOMACH

Well grown cats were decerebrated by pithing without the previous induction of anaesthesia. Tracheotomy was performed immediately afterwards, a tube placed in the trachea and artificial respiration maintained. A cannula was introduced into the right carotid artery and connected with a mercury-manometer. By this means a constant blood pressure was obtained without the disturbing influence of an anaesthetic. The stomach was then exposed by a small

<sup>1.</sup> Fraser, *Transactions Royal Society, Edinburgh*, Vol. XXXV, Part IV, 1890; Vol. XXXVI, Part II, 1891.

\* M.L.D. = Minimum lethal dose.

incision in the linea alba about two cms. long, and five c.c. of the undiluted standard tincture was injected with a fine hypodermic needle through its wall. Short records of the blood pressure were then taken every five minutes.

In the case of digitalis no effect was noticed on the blood-pressure during two hours ; with squill after the same lapse of time, there was also no rise. But with strophanthus, even after one hour's action, the heart went into the condition known as *delirium cordis*, and after beating very rapidly and irregularly for thirteen minutes it suddenly ceased to act, and after death was found in fibrillary twitchings. There was no rise in blood-pressure at any time.

One is bound to conclude, therefore, that tincture of strophanthus, whether given by the mouth or hypodermically, is very much more toxic than the other two drugs in this group. And here is an explanation of a clinical effect not infrequently observed, that a case of cardiac disease which is not improving under digitalis, may immediately improve when tentatively placed upon strophanthus. The patient thus receives a dose five or ten times as potent as that he was previously taking.

The problem of absorption by the gastric mucous membrane is of considerable importance. Under normal conditions, no water or dilute saline solutions appear to be absorbed by the stomach, but volatile substances, such as alcohol or arsenic, are absorbed with ease ; as they are from all parts of the intestinal canal. It might therefore be objected that the digitalis and squill had little chance of being absorbed, and this is no doubt true ; the experiments are only of comparative value. Nevertheless they show conclusively that strophanthus, even under these conditions, was absorbed, although there was no evidence of the absorption of either digitalis or squill. I wish then to draw attention to the result that not only is strophanthus much more toxic than the other two drugs, but also that it is absorbed with at least equal facility from the mucous membranes.

#### CHANGES IN THE STOMACH WALL

These experiments also showed certain changes in the gastric mucous membrane. Digitalis caused an intense congestion and

corrosion of the tissue, which in one place was necrotic. Squill had a somewhat similar but much less marked effect. Strophanthus made hardly any impression at all. It must be remembered, however, that the stomach was subjected to the action of digitalis and squill for a period of time nearly twice as long as that during which strophanthus was acting.

The members of the digitalis group, when introduced in suitable solution into the eye, eventually give rise to anaesthesia of the conjunctiva and cornea. Digitalis and squill cause intense inflammation before the stage of anaesthesia is reached. Strophanthus, however, produces a local anaesthesia in about fifteen minutes with comparatively little irritant action. It was tried, indeed, as a local anaesthetic in ophthalmic practice before the introduction of cocaine. Further, it is a well-recognised clinical observation that digitalis and squill may give rise to emesis and purging ; squill has, indeed, been prescribed as an emetic. Strophanthus induces intestinal disorders much more rarely. The expectorant action of squill is explained as a reflex secretion from the bronchioles, brought about by irritation of the gastric mucous membrane. If this explanation be accepted, digitalis may be assumed to be a more potent expectorant than squill.

Hence the three drugs under consideration all possess an irritant action, which bears no direct relation to the toxicity ; that is to say : strophanthus, which is the least irritant, is the most toxic ; and digitalis is the most irritant, and has about the same toxicity as squill.

#### EXPERIMENTS ON THE MAMMALIAN HEART

Having indicated the relative strength of these drugs and certain of their characteristics, I now proceed to the consideration of a comparison of their respective actions on the mammalian heart. For this purpose I employed a modification of Langendorff's method, by which, by perfusion of an excised heart through the coronary vessels, may be obtained, for some hours, a free and regular heart-action. Healthy young rabbits, from six weeks to three months of age, were used for the greater number of these experiments, but the effects were obtained equally well on the hearts of other mammals.

*Apparatus.* The apparatus employed for these perfusions is of the simplest description. It consists of an ovoid glass vessel with openings above and below, the lower opening terminating in a nozzle. To this a piece of rubber pressure tubing is fixed, which, in its turn, is connected with an S-shaped piece of glass tubing. This, before each experiment, is loosely packed with glass wool, and this serves the purpose of filter. The pressure tubing is also provided with a T-piece, which can be opened and closed at will, and may be employed for the introduction of drugs.

To the lower end of the filter a further piece of pressure tubing is fixed, serving to connect the cannula to which the heart is attached ; this tubing has a regulating screw, so that the supply of fluid to the heart can be increased or diminished at will. The upper opening in ovoid flask is fitted with a rubber cork, through which pass two glass tubes ; the one, passing to the bottom of the vessel, serves for the introduction of oxygen ; the other only just passes through the cork, and is connected with a mercury valve for regulating the oxygen pressure. The flask is surrounded by a water-jacket, by means of which its temperature can be kept constant. The cannula is of a bulb form, and is provided with a lateral tube, through which a delicate thermometer is passed.

The fluid used for perfusion was a modified Ringer's solution, as suggested by Sherrington, to which a little dextrose was added.

In experimenting by means of this apparatus with members of the digitalis group, I chiefly employed the standard tinctures diluted 1 in 2500 times. I also observed the effects of introducing single doses into the side tube, and of varying the strength of the dilution of the perfused drugs.

#### RESULTS

*The Rate of the Heart-Beat.* The first noticeable effect of the action of these drugs on the heart is on the rhythm. The heart beats more slowly. This effect begins a few minutes after the introduction of the drug, and lasts during the first stage of the experiment, that is, until the tonus has commenced to rise considerably, when the heart-

beat again quickens, and may enter into the condition known as *delirium cordis*. This slowing is mainly diastolic, but as I propose to show presently, a small portion of it is systolic, that is to say, not only is diastole prolonged, but also the length of systole is increased, although the increase is not in any way comparable to that of the diastole. In these experiments it is obvious that the slowing must be induced by an action on either the vagal mechanism or the musculature of the heart. This point can be readily determined by eliminating the vagus-action by a previous exhibition of atropine. In such experiments it was found that the diastolic slowing, which is the characteristic effect of all the members of this group of drugs, did not occur to any extent; and often an atropinized heart, under the influence of digitalis, squill, or strophanthus, will maintain its rate almost unaltered until the second stage, that of acceleration and arrhythmia, is reached—that is, all through the therapeutic stage. It is therefore obvious that the greater part of the diastolic slowing is due to excitation of the vagal terminations in the heart.

Of the three drugs under investigation, compared in equal doses of their respective tinctures, strophanthus is the most effective in producing slowing of the heart-beat; squill comes next, and digitalis has the least effect on slowing (see figs. 6, 7 and 8). After the administration of these drugs, the average diminution in rate during the therapeutic stage, calculated from twenty-four experiments, is:—

1. Strophanthus	...	52%
2. Squill	...	42%
3. Digitalis	...	30%

In other words, it is to be expected in such experiments as these that, given a normal heart rate of 100 beats per minute, the slowest rate reached in the therapeutic stage will be 48, 58, and 70, after the exhibition of strophanthus, squill, and digitalis, respectively. This is represented in the subjoined diagram, in which each pair of columns graphically represents the difference in the rate of heart-beat after the administration of the three drugs respectively.

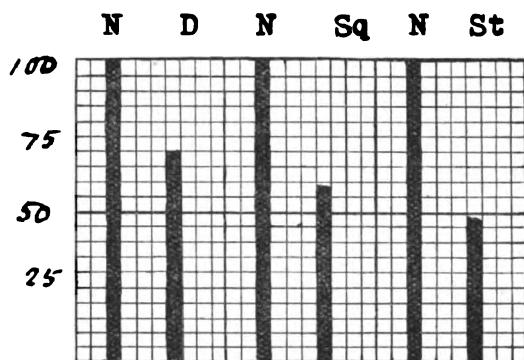


FIG. 1. Heart-beats per minute.

N = normal mean rate. D = slowest mean rate after perfusion with Digitalis.

Sq. = " " " " Squill.

St. = " " " " Strophanthus.

It must not be forgotten, however, that although these drugs are being administered in equal doses according to the British Pharmacopoeia, such doses are not proportional to the strengths of the tinctures. As I have previously shown, strophanthus is 8-10 times more toxic than the other two drugs, and if the three were given in proportionate doses, then squill would evidently be seen to be by far the most efficient drug in slowing the heart-beat. The systolic slowing is not of much significance in this connection, and will be further considered under the next heading.

Very occasionally the heart may show an initial acceleration after the administration of any of these drugs, more especially in the case of strophanthus (*c.f.*, fig. 7). This effect never lasts longer than a few minutes, and is generally associated with an increased flow of fluid through the coronary vessels. The explanation of this, which I suggest, is that these drugs exert two main actions on the heart ; first, they slow the beat ; and secondly, they increase the irritability of the cardiac muscle, and thereby increase the force of the beat. In certain experiments the irritability is increased, just at first, to such an extent that the vagal mechanism is unable to hold the heart in check, and hence the quickening in the rate of the heart. An acceleration from such a cause follows in the usual sequence of events in the

second stage of poisoning by these drugs, when the heart enters into *aelirium cordis*. The associated increase in the flow from the coronary vessels must be explained by the increase in liberated metabolites, as a result of the augmented cardiac muscle work. It is the same effect that Gaskell observed during activity of skeletal muscle—the vessels dilate as a result of the local liberation of metabolites.

With regard to the time taken by the action of the three drugs before the greatest slowing is reached, the averages are as follows :—

1.	Strophanthus	...	15	minutes
2.	Squill	...	24	"
3.	Digitalis	...	63	"

It is thus seen that digitalis takes by far the longest time to produce the maximum slowing.

*Experiments on the Atropinized Heart.* Excised hearts are perfused in the usual manner, and when the action is regular and constant, a minute dose of sulphate of atropine, 0.0025 grm. in warm saline solution, is introduced into the apparatus through the side tube.

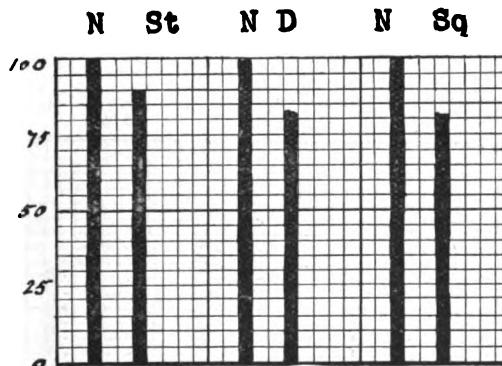


FIG. 2. Heart-beats per minute

N = normal mean rate of atropinized heart ; St. D. Sq. = slowest mean rate after the introduction of Strophanthus, Digitalis, and Squill, respectively.

This immediately produces some quickening, which after a few minutes subsides and the heart resumes its previous rate of action. The drug whose action is to be observed is then perfused as in the

previous experiments, but different results are obtained. The slowing is much less, and the time taken to produce the greatest slowing is prolonged. Thus if the rate before the introduction of the three drugs strophanthus, digitalis, and squill, is 100 per minute, the lowest rate caused by each is 89, 82, and 81, respectively (see Fig. 2).

The period of time which elapses before the greatest slowing is produced is 120 minutes for digitalis and 30 minutes each for strophanthus or squill.

By comparing the results of the action of the drugs on the atropinized heart with those obtained when the heart is not previously atropinized, the following tables have been produced.

TABLE A

Drug	Percentage decrease in rate of heart-beat in beats per minute			
	Without atropine	With atropine	Without atropine	With atropine
Digitalis	...	...	30 %	18 %
Squill	...	...	42 %	19 %
Strophanthus	...	...	52 %	11 %

Fig. 3 is a diagrammatic representation of above table.

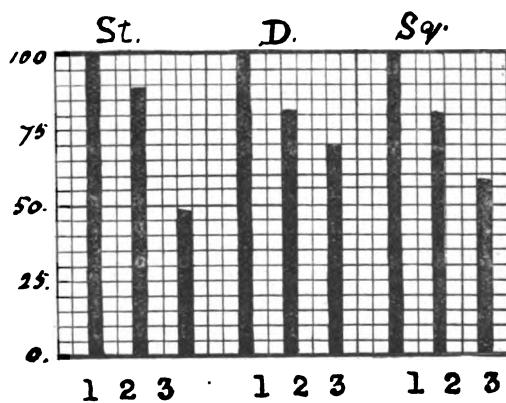


FIG. 3. Beats per minute

1. Normal mean rate.
2. Lowest mean rate caused by drug on atropinized heart.
3. Lowest mean rate caused by drug on normal heart.

In the second table (B) the period of time in minutes before greatest slowing is produced is shown.

TABLE B

Drug	Without atropine	With atropine
Digitalis ...	63 minutes	...
Squill ...	24 "	...
Strophanthus ...	15 "	...

It thus appears that the drugs, acting on the atropinized heart, cause very much less slowing than they do when acting on the normal heart; and also that the time elapsing before this slowing reaches its maximum is doubled in the case of digitalis and strophanthus, and prolonged in the case of squill. The inference, therefore, is that this group of cardiac tonics directly excites the terminations of the vagus in the heart, and that this is the chief cause of the slowing. (Compare figs. 6, 7, and 8, with figs. 9, 10, and 11, respectively.) Considerable difficulty was experienced in administering a dose of atropine sufficient to paralyse the vagal terminations without causing the death of the heart. The amount usually administered (gms. 0.0025) sometimes caused a very rapid action, followed by death, in diastole; but if given slowly and carefully it was eventually found to be sufficient to paralyse the nerves without killing the muscle or affecting the accelerator mechanism. Fraser, in his work on *Strophanthus hispidus*, found that 'the previous administration of atropine did not prevent, nor indeed in any conspicuous manner modify, the production by strophanthus of the changes in the heart's contractions which have been seen to follow its application to frogs to whom (*sic*) no atropine had been administered.' But, on the contrary, with rabbits he found that 'the administration of atropine succeeded in lessening its (strophanthus) effect on the rate of the pulse.'

*The Quality of the Heart-beat*—It is well known that drugs which excite the ordinary cardiac muscle increase its tonus and both the force and amplitude of its contraction. Examples of such drugs are barium

and veratrine. The increased tonus, however, is not observed with these drugs provided that they are administered in moderate doses, because of the prolonged state of diastole from excitation of the vagus, Squill is by far the most effective drug for augmenting the force of the cardiac systole without increasing the tonus. Both this drug and digitalis, during the first or therapeutic stage of their action, cause more complete contraction and more complete relaxation of the cardiac muscle. Strophanthus causes a very complete systole with no corresponding increased relaxation. (*Cf.* figs. 6, 7, 8).

Fraser finds that 'with a considerable or a large dose of strophanthus the systolic type of change is produced, in which by a direct action on the heart's muscle the capability to relax is diminished, until diastole becomes impossible, and the heart ceases to pulsate, with the ventricles so thoroughly contracted that its cavity is almost effaced.' With small doses, he describes a diastolic type of change, and says that 'whatever be the type, great increase occurs in the movements of the heart by exaggeration of expansion as well as of contraction. This occurs only temporarily where the type is systolic, but throughout, or nearly throughout, the action where the type is diastolic.'

This action of strophanthus may occur in frogs, but it is certainly not the case with the mammals ; because in the perfusion experiments, although the dose received by the heart is small, no increased relaxation was observed in any case ; whereas in an experiment in which a large dose, 1 c.c. of tincture of strophanthus, was introduced into the side tube, there was at first marked dilation and slowing, soon followed by great rise in tonus, and an increase in force and rate of the heart. The latter experiment, however, is hardly physiological, because such a large dose of strophanthus as is here administered can never reach the heart at any one time in man. The perfusion experiments, in which a small amount of this drug is allowed to act over a prolonged period, more nearly approach the conditions which obtain in life.

Fig. 4 shows the relative increase in the excursion of the lever attached to the heart after exhibition of the three drugs.

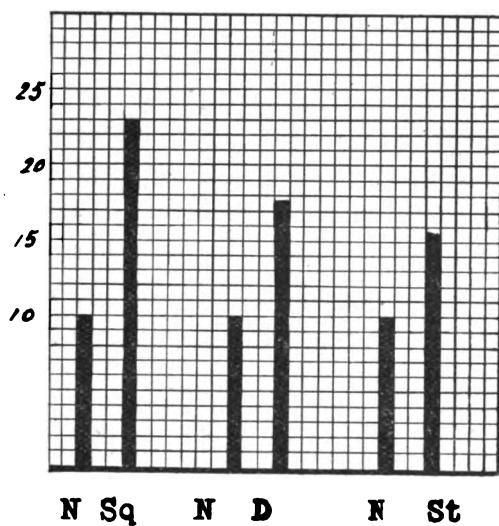


FIG. 4. Showing the relative increase of force of heart-beat.

N = normal excursion of lever ; Sq. D. and St. = excursion of lever after Squill, Digitalis, and Strophanthus respectively.

The average increase in excursion is : after squill 13, after digitalis 7.6, and after strophanthus 5.5, the initial excursion being reduced in each case to 10, for the purpose of comparison.

All three drugs cause death of the heart in the systolic phase. This may be said to be invariable ; in twenty-six out of twenty-seven experiments the ventricles were tightly contracted at death, the one exception occurring during the perfusion of digitalis, when the heart died suddenly during diastole, after three hours' action (see fig. 6). And without doubt, in this case, had atropine been given, the heart would have continued to beat till death occurred in systole.

Strophanthus usually causes the heart to die suddenly when the tonus has been greatly increased and the relaxation is very slight. The heart actually stops during the diastolic pause ; and the ventricles then very slowly contract up without beating, until the cavity is obliterated. Digitalis and squill nearly always cause the condition known as *delirium cordis*, preceding death in systole. The heart passes suddenly into rapid irregular twichings which last five or six

minutes to an hour. The ventricles cease in tonic contraction and the auricles continue to beat feebly.

Besides increasing the force of the beat, these drugs also prolong the beat, that is to say the heart remains in systole for a longer period than normal. This effect is not seen in the majority of the tracings, because the object has been rather to show up the increased force of the beat and the prolonged diastole, but it is shewn quite easily when the tracings are taken on a more quickly moving drum. It is most pronounced with squill, and least with strophanthus. With squill it may at times be so pronounced that nearly all the slowing is systolic.

*Relative Toxicity on the Heart Muscle.*—The relative toxicity of the three drugs on the heart muscle is determined by comparing the lengths of time elapsing between the introduction of the drug and the death of the heart.

Drug perfused	Strength	Time elapsing before death	Ratio of toxicity
Tr. Digitalis . . .	$\frac{1}{2500}$	142 minutes	1
Tr. Scillae . . .	$\frac{1}{2500}$	93 , ,	1.5
Tr. Strophanthi . . .	$\frac{1}{2500}$	42 , ,	3.4

This is not of course a true standard of toxicity; for it does not necessarily follow that because one drug is twice as toxic as another it kills in half the time. The table supports the experiments on standardization made on the frog, and shows how much more toxic strophanthus is than digitalis.

On the atropinized heart strophanthus is apparently not so toxic, while digitalis and squill have nearly the same toxicity as on the normal heart, both absolutely and relatively.

Drug perfused	Strength	Time elapsing before death	Ratio
Atropine sulphate . . .	gm. 0.0025		
Tr. Digitalis . . .	$\frac{1}{2500}$	150 minutes	1
Atropine sulphate . . .	gm. 0.0025		
Tr. Scillae . . .	$\frac{1}{2500}$	100 , ,	1.5
Atropine sulphate . . .	gm. 0.0025		
Tr. Strophanthi . . .	$\frac{1}{2500}$	60 , ,	2.5

*Action on the Coronary Vessels*—From a consideration of the literature on the subject it has become evident to me that the blood supply to the heart itself has not received adequate attention. In the standard works dealing with cardiac disease, although gross disease, such as atheroma is fully discussed, the state of the coronary arteries in such conditions as hypertrophy and atrophy of the heart, in Bright's disease, and in cases of high blood-pressure, is scarcely noticed. It is obvious that the condition of the coronary arteries and the blood-supply of the heart muscle must be of paramount importance, both in the causation, and as a factor in the treatment of cardiac disease. It has been shown by Schäfer<sup>1</sup> that the coronary vessels have no nerve supply, and this observation has been confirmed by Elliot<sup>2</sup> who showed that adrenalin, pilocarpine, and muscarin, drugs which excite nerve-endings, do not appreciably alter the calibre of these vessels. And on consideration, it is evident that it is essential that they should not be innervated and so constrict under conditions which influence the general circulation; herein possibly lies an important factor in the cardiac hypertrophy in interstitial nephritis. In this condition the peripheral and splanchnic vessels constrict, owing probably to the need of increasing the blood-pressure in order that more blood may circulate through the excretory apparatus which is diminished in extent by disease. But the coronary vessels do not constrict, and as by the greater pressure in the sinuses of Valsalva, more blood is forced through the heart muscle, it becomes more vascular and therefore hypertrophies in just the same way as any other organ will hypertrophy when its blood supply is permanently increased.

In order to examine the action of these three drugs on the coronary arteries, I adopted the following method of estimating the flow of fluid through these vessels. Perfusion experiments were performed as before described, and the amount of fluid passing through the coronary arteries during one minute was measured in a cylindrical glass graduated vessel at intervals of five minutes. The measurements in cubic centimetres were charted, and curves obtained for each drug. (See Fig. 5).

1. Schäfer, *Proc. Brit. Assoc.*, Cambridge, 1904.

2. Elliot, *Journal of Physiology*, XXXII, p. 401, 1905.

These experiments show that strophanthus has little or no effect—there is a diminution of 0.2 c.c. in sixty minutes. Digitalis causes some gradual decrease in the flow through the vessels—0.6 c.c. in the first sixty minutes, and 1.1 c.c. in three hours. Squill still further decreases the flow by 0.9 c.c. in the first sixty minutes, and 1.5 c.c. in three hours. It acts more quickly than digitalis. These results are here shown in tabulated form :—

Drug	Average decrease in flow per minute		Total decrease in first 60 minutes	Total decrease during action
Squill	...	...	0.0089 c.c.	0.9 c.c.
Digitalis	...	...	0.006 c.c.	0.6 c.c.
Strophanthus	...	...	0.0036 c.c.	0.2 c.c.

From these results it appears that although, as has been before mentioned, the coronary vessels are not innervated, yet squill and digitalis cause some constriction of them. How can this be accounted for? It might be urged that the vessels were constricted by direct compression from the heart muscle owing to increase of the tone of the cardiac muscle. Against this is the action of strophanthus; for, although it causes even more increase in tonus than the other two drugs, yet the measurement of the fluid perfusing the coronary vessels at the time of death shows hardly any difference from that of the fluid passing through before the drug was administered. Possibly by their irritant action, squill and digitalis cause contraction of the muscular coat of the coronary arterioles; in fact, there seems to be no other explanation of their action. And this is also in harmony with what we know concerning the action of these drugs on other forms of plain muscle.

When injected into the circulation all three drugs cause a rise in blood-pressure—squill the greatest and strophanthus the least—strophanthus having very little action even on innervated arterioles. Squill is recognized clinically as a potent diuretic, this action being due to increased cardiac output combined with a rise in blood-pressure, in producing both of which factors its acts more powerfully than digitalis.

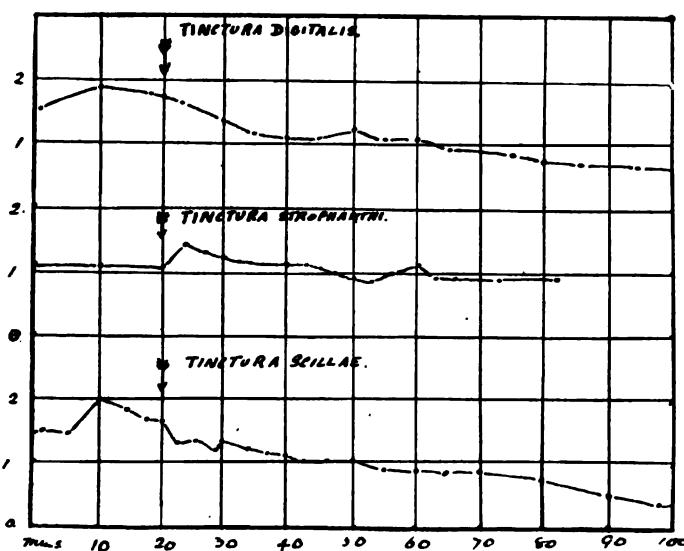


FIG. 5. Graphic representation of three perfusion experiments on young rabbits.

The coronary arteries were perfused with Ringer's solution. The ordinates equal outflow in c.c. per min., and the abscissae time in minutes. Perfusion with the drug, which in each case represents a strength of 1 in 2,500 of the official tincture, commenced at the arrows. Squill and digitalis constrict the vessels about equally, squill being a little the more active. Strophanthus has hardly any action ; the initial dilatation it induces is due to products of metabolism from the increased cardiac activity. Perfusion pressure = 25 mms. of mercury.

#### CONCLUSIONS

In reviewing the results obtained from these experiments, the most striking feature is the superiority of squill over digitalis and strophanthus as a cardiac stimulant. The commercial preparations of this drug show less variability in strength, and so they are more trustworthy. Squill has a more constant action than digitalis : it has, moreover, a greater effect in increasing the work done by the heart, and more effect on the vessels ; it is also less irritant to the gastric mucous membrane. Squill is popularly regarded as a stimulating

expectorant, and is a common ingredient of cough mixtures ; but its well-marked cardiac action is very frequently overlooked. No doubt its popularity as a so-called expectorant is chiefly due to the almost invariable custom of prescribing it along with other expectorants, and relief is afforded by its action in allaying the distention of the right side of the heart, so common a condition in bronchitis and other pulmonary affections. But with regard to its reflex expectorant action from irritation of the stomach, digitalis should prove as effective, if not more so, for it is a stronger gastric irritant. I should like to enter a plea for the more extended use of squill in place of digitalis in cases of heart failure : it acts more quickly, more effectively, and does not cause any more, if as much, gastro-intestinal derangement. Every precaution which is observed when digitalis is properly administered should also be observed in the use of squill. It should not be given for many days without intermission, and a close scrutiny must be kept on the pulse rate and on the amount of urine passed. A pulse below 60, and a rapid diminution in the quantity of urine are indications for its withdrawal.

With regard to digitalis, it is a common experience amongst practitioners of medicine that its preparations are often untrustworthy. Dixon and I have shown that some tinctures, at any rate, are only about one third the strength of others. This may be due to the quality of the leaves selected (these show great variation in percentage of glucosides), or to an inexact and careless method of preparation, or to the age of the tincture. The solution of the difficulty is to be found in physiological standardization of these drugs. Preparations should be placed on the market, suitably labelled, stating definitely that at a given date a certain dose killed a frog of so many grams within a limited time. The standard could be fixed, as in these experiments, so that  $2\frac{1}{2}$  minims kills a 20 gramme frog within three hours, and the tincture diluted accordingly. Experiments are being conducted at Cambridge with a view to ascertain how long these tinctures retain their potency.

Strophanthus is a dangerous drug. The Pharmacopoeial dose is 5-15 minims, like those of digitalis and squill, yet it is 8-10 times as

toxic as either. It has a powerful action on the heart, and is apt to cause sudden death without warning. In small doses it is no doubt valuable in cases of heart failure associated with marked arterial degeneration, for its action in constricting the vessels is very slight. The dose in the Pharmacopoeia should be reduced. In this connection it is interesting to notice that Dr. Sansom, in his article on *Disease of the Mitral Valve*, in Clifford Allbutt's System of Medicine, recommends tincture of digitalis in doses of 5-30 minims, and tincture of strophanthus in doses of 2-10 minims, every 4 hours. He says 'there are some probabilities that it (strophanthus) may lead to sudden death in the course of its administration for heart disease (Gottlieb) . . . . I cannot doubt that the protracted injudicious administration of digitalis and strophanthus has often been productive of dangerous and fatal results.'

#### SUMMARY OF RESULTS

1. When standard tinctures of the three drugs, digitalis, squill, and strophanthus, are freshly prepared strictly according to the British Pharmacopoeia, then, supposing the toxicity of digitalis to be represented by one, squill will also represent one, but strophanthus nine or ten.

2. It is essential that these drugs should be standardized. The only way by which this can be done effectually at the present time is the bio-chemical method.

3. Digitalis, squill, and strophanthus all have a specific action on the heart, which can be divided into two stages; a first stage characterized by stimulation of the cardiac muscle and of the vagal nerve endings; and a second, in which the tonus is much increased, and the rate accelerated by the extension of the action on the muscle to the excito-motor area of the heart.

4. Tincture of squill differs from the other drugs in its action upon the heart in the following ways:—

(a) In therapeutical doses it increases the force of contraction to a considerably greater extent than either digitalis or strophanthus. Not only is systole more complete and prolonged but diastole also.

- (b) It slows the heart-beat more than digitalis, and, considering its relative toxicity, more than strophanthus. This, however, is its effect only so far as the peripheral **vagal** mechanism is concerned.
- (c) It produces more vaso-constriction of the coronary **vessels** than either digitalis or strophanthus.

5. Tincture of digitalis is less efficient as a cardiac stimulant (that is a drug which increases the cardiac output per beat) than squill, in that it has not the same effect in increasing the energy of contraction of the heart. Moreover, it is not possible by altering the relative dosage of digitalis to produce a stimulant effect in any way comparable to that of squill. Digitalis has less peripheral effect on the **vagal** mechanism than squill.

6. Tincture of strophanthus being eight to ten times more toxic than the other two drugs, it is hardly fair to compare it with equal quantities of them. Such comparisons, however, serve to confirm the experiments made on frogs, showing the greater activity of this drug. With regard to its effect on the force of the heart-beat, strophanthus occupies a position considerably below squill and rather less than digitalis. It slows the heart more than either of the other two tinctures in equal doses. In contra-distinction to digitalis and squill, strophanthus has practically no action on the coronary vessels. At first it slightly increases the outflow from the coronary veins the result of metabolites set free by the increased work of the heart. This drug is very apt to cause sudden death of the heart without previous excitation of the excito-motor area.

7. After previously atropinizing the excised heart, perfusion of these drugs causes very little decrease in the heart-rate. Their local action on the peripheral **vagal** mechanism is a large factor in the production of the usual slowing seen during their exhibition to patients.

8. To mucous membranes digitalis is the most irritant, then comes squill, while strophanthus has very little irritant action.

9. In any case in which it is desirable to raise the blood pressure and stimulate the heart, squill should be used in preference to the other two drugs.

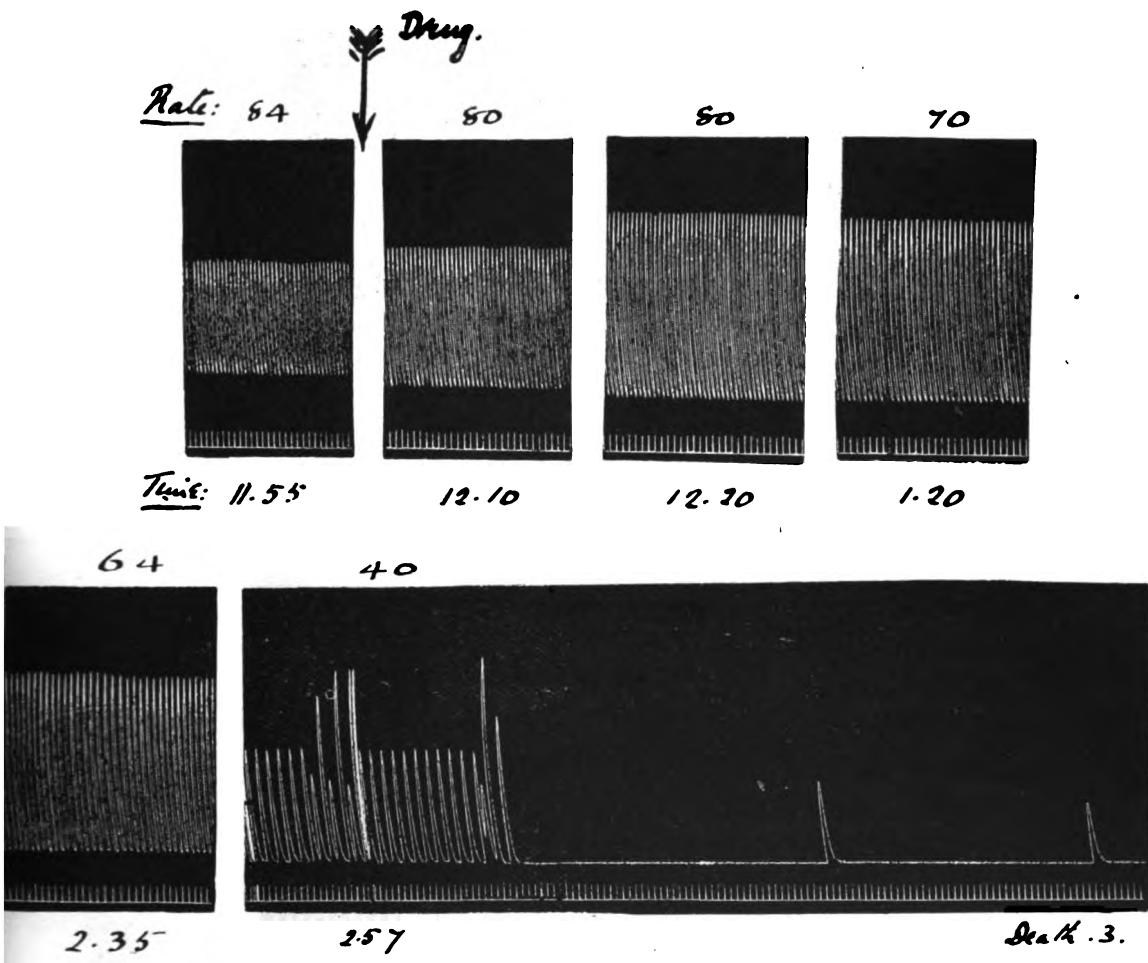


FIG. 6.—Rabbit's heart: perfusion with Ringer's solution. At 11.55, a piece of normal tracing is shown; at 12, 1 in 2,500 Tr. Digitalis was substituted, and tracings of the heart-beat are shown at various subsequent times. Death here occurs in diastole, which is very exceptional. Upstroke = systole. Time = secs.

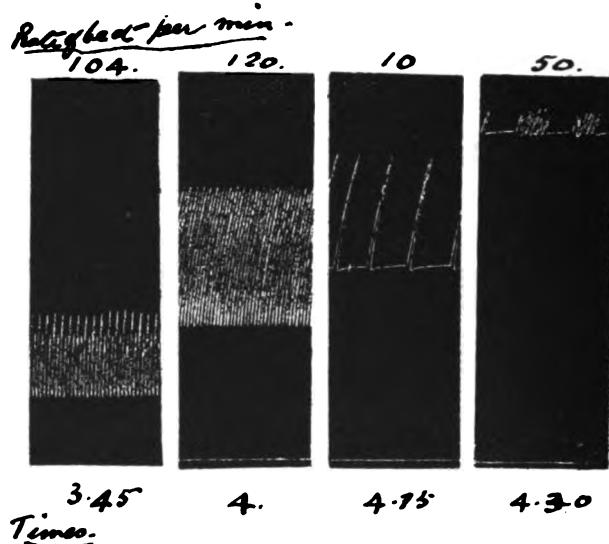


FIG. 7.—Rabbit's heart : perfusion with Ringer's solution. At 3.45, a piece of normal tracing is shown ; at 4.0, 1 in 2,500 Tr. Strophanthi has been substituted for 15 mins. ; at 4.15 and 4.30, later effects are shown.

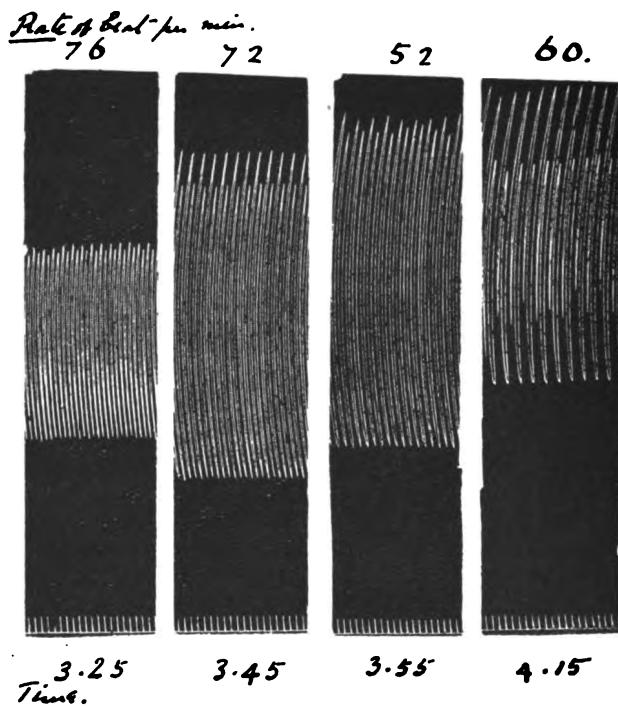


FIG. 10.—Rabbit's heart : perfusion with Ringer's solution. At 3.15, gm. 0.0025 atropine sulphate was added ; at 3.25 a tracing of the atropinized heart-beat is shewn ; then 1 in 2,500 Tr. Strophanthi was substituted.

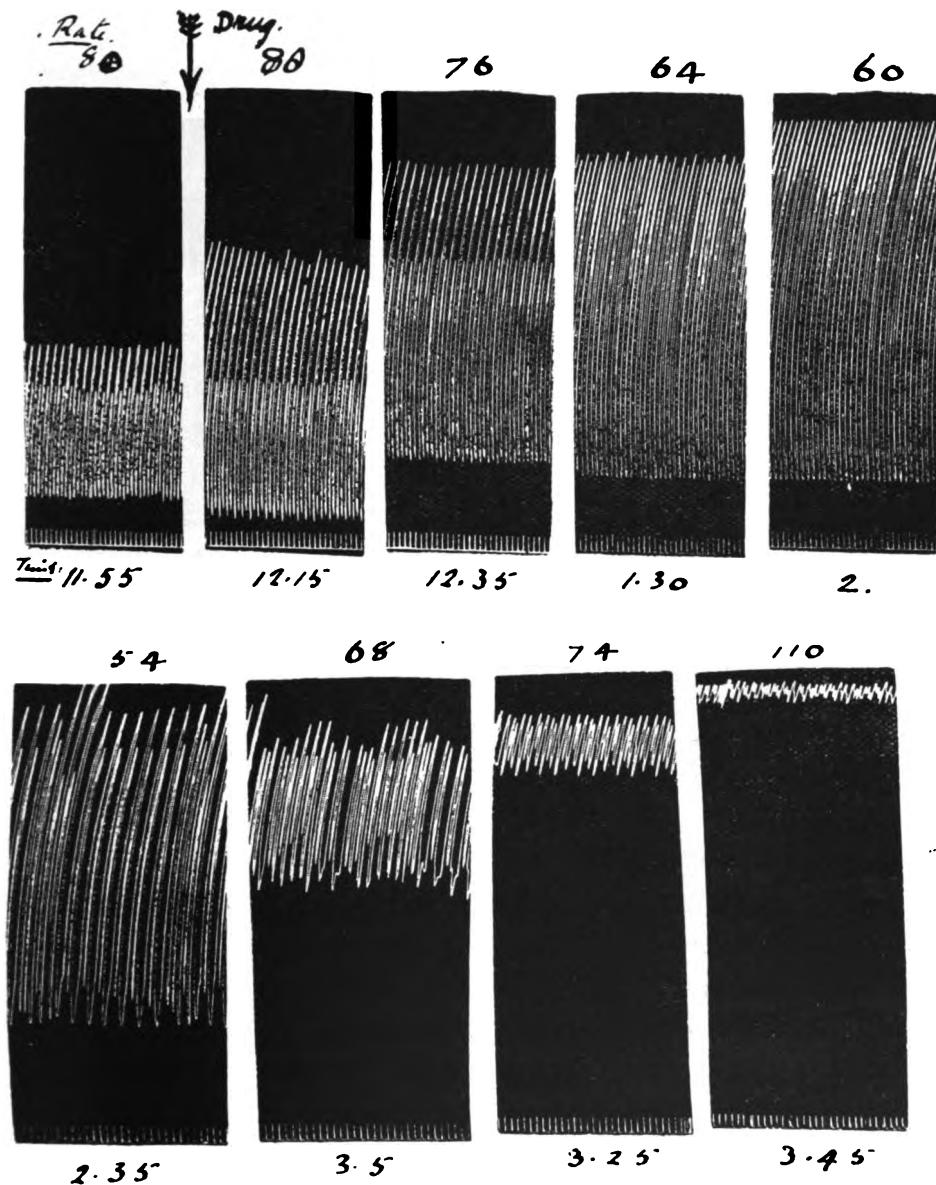


FIG. 8.—Rabbit's heart: perfusion with Ringer's solution. A piece of normal tracing at 11.55 is shown; at 12.0, 1 in 2,500 Tr. Scillae was substituted—the subsequent tracings show the effects on the heart-beat.

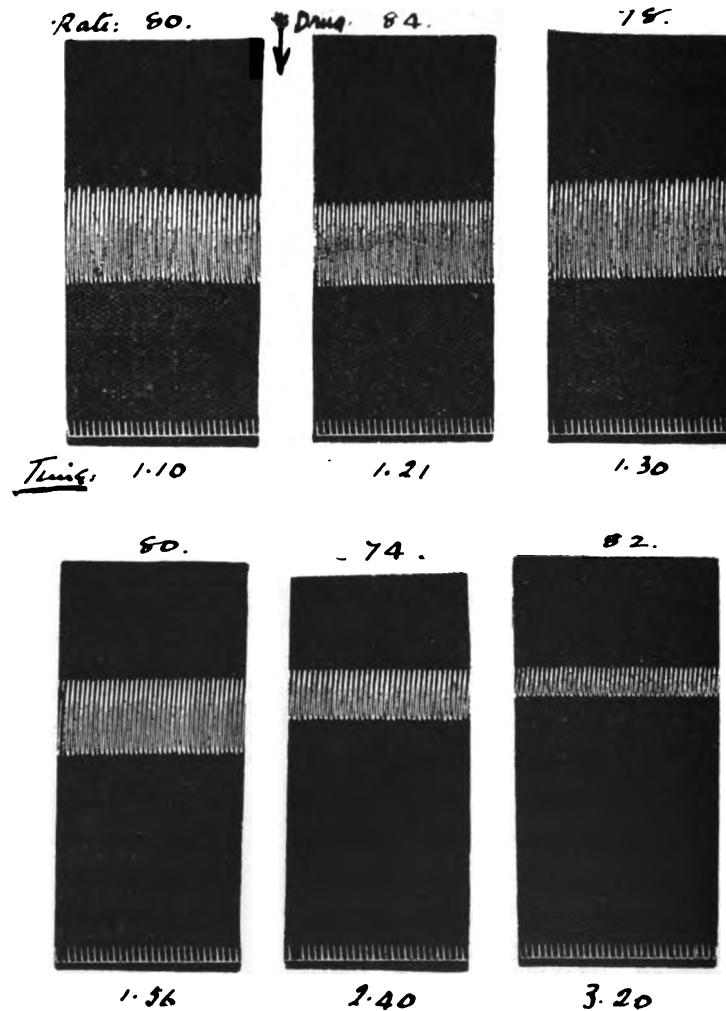


FIG. 9.—Rabbit's heart : perfusion with Ringer's solution. At 12.35, gm. 0.0025 atropine sulphate was added ; at 1.10, a tracing of the atropinized heart-beat is shown ; at 1.15, 1 in 2,500 Tr. Digitalis was substituted, and the effects are shown at various intervals.

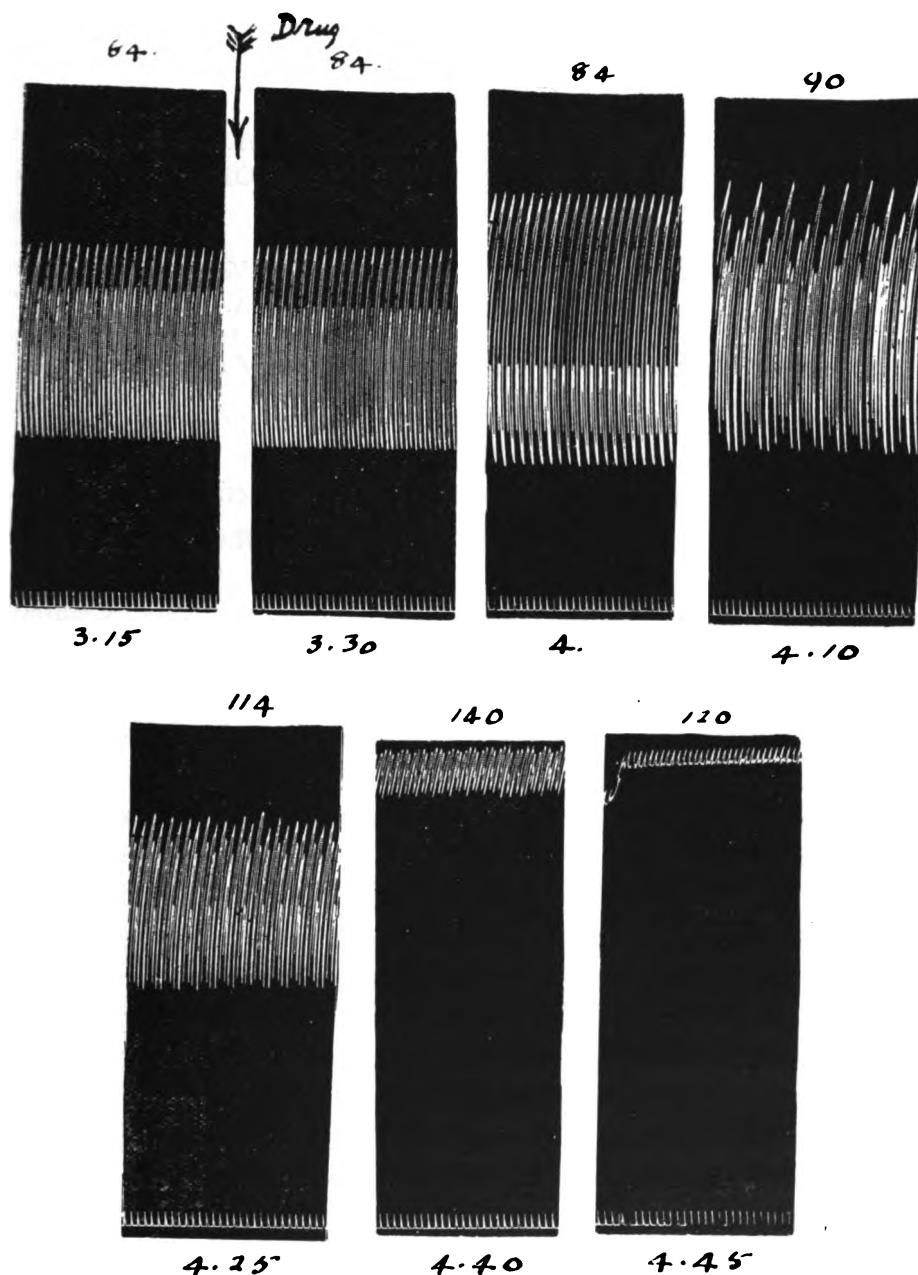


FIG. 11.—Rabbit's heart: perfusion with Ringer's solution. At 3.0, gm. 0.0025 atropine sulphate was added, and a tracing is shown at 3.15 of the beat of the atropinized heart; at 3.20, 1 in 2,500 Tr. Scillae was substituted.

THE ACTION OF ACIDS AND ALKALIES AND OF ACID,  
ALKALINE AND NEUTRAL SALTS UPON THE TAD-  
POLE OF 'RANA TEMPORARIA'

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Many observations have been made upon the action of inorganic salts and variations in their concentration upon living cells. We shall not attempt a complete review of the literature of the subject, but will confine ourselves to the consideration of those observations which are more nearly related to our own experiments.

Ringer and Phear<sup>1</sup> stated that distilled water was toxic for tadpoles, and tested the effects of various chemicals in lessening its supposed harmful action. Locke<sup>2</sup>, however, showed later that distilled water is not necessarily fatal, but that if the least trace of copper be present the solution acquires toxic properties. He further showed that this action of copper can be destroyed in various ways, and suggested that the results of Ringer and Phear might be due to the presence of traces of copper, and that the actions of the agents employed consisted in rendering the copper inert. Ringer<sup>3</sup>, in a later communication, confirmed Locke's work, showing that distilled water, free from traces of copper and other metals, had no toxic effect on tadpoles.

Loeb and Gies<sup>4</sup>, using the eggs of *Fundulus*, found that a single salt added to distilled water stopped the development, whilst controls in distilled water grew perfectly, but that a second salt of different valency, when used in a suitable ratio, counteracted this effect.

1. *Journal of Physiology*, Vol. XVII, 1895, p. 423.
2. *Journal of Physiology*, Vol. XVIII, 1895, p. 319.
3. *Journal of Physiology*, Vol. XXII, 1897, *Proceedings*, p. 14.
4. *Arch. f. d. ges. Physiol.*, Vol. XCIII, 1903, p. 246.

Loeb<sup>1</sup> also studied the effect of altering the reaction of the medium upon the growth of *Tubularia*. He found that regeneration was hastened on adding a trace of alkali to an artificial sea water in which the stems were placed. Loeb<sup>2</sup> also found that the addition of minute traces of alkali to sea water hastened the growth of *Echinus* eggs.

The observations of Moore, Roaf, and Whitley<sup>3</sup> upon altering the reaction of natural sea water, in which the fertilized eggs of *Echinus esculentus* were growing, show that alkalies and alkaline salts favour cell division when added in sufficiently small amounts, but that on increasing the strength the growth becomes arrested, whilst acids and acid salts possess no favouring action but inhibit the development of the eggs throughout.

In the present experiments we have studied the effect of adding various chemicals to a solution in which the tadpole can live for some length of time. For this purpose we used the tapwater supplied to this laboratory, after allowing it to run for some time in order to get rid of the water which had been standing in contact with the brass taps and other connections.

The effect of varying the reaction has been studied as well as the action of neutral salts containing similar ions to those used for altering the reaction. The organisms used were the larvae of *Rana temporaria*, which were all of the same age and probably came from a single spawning.

#### EXPERIMENTAL METHODS.

The experiments were conducted in flat earthenware dishes to expose a large surface for the exchange of gases necessary for the respiration of the animal. In each of these dishes was placed 300 c.c. of a mixture consisting of tap water and the requisite amount of standard solution to give the desired concentration. These solutions were changed every few days to prevent alteration of the strength by evaporation and by the excreta of the animals.

1. *Arch. f. d. ges. Physiol.*, Vol. CI, 1904, p. 340; also, *University of California Publications Physiology*, Vol. I, 1904, p. 137.

2. *Arch. f. Entwickelungsmechanik d. Organismen*, Vol. VII, 1898, p. 631.

3. *Proc. Roy. Soc. B.*, Vol. LXXVII, 1905, p. 102.

In nearly all cases six tadpoles were used, but preliminary experiments were often performed in which a single tadpole was used to determine the rough limits of concentration within which the tadpoles could live. By these means we obtained the concentrations necessary to kill the organisms. The rate of growth was studied in weaker solutions by observing the weight at the end of the same period of growth in the different solutions, and also by noting the time at which the four-legged stage was reached.

The weights were obtained by removing the excess of moisture from all the tadpoles grown in one dish and then weighing them. Each lot was treated in exactly the same manner and the weight of the tadpoles was divided by the number of tadpoles remaining alive, by which means the average weight of the tadpoles in each solution was obtained. In this series the experiments were all started on the same day and were kept going for the same length of time, thus the results are strictly comparable [Table VII (a)].

The solutions of the chemicals to be tested were made up in distilled water, and by adding a definite amount to tap water the requisite strength was obtained. The acids and alkalies were standardized by titration in the presence of an appropriate indicator, the carbonates were weighed and then titrated, whilst the phosphates were weighed, titrated, and finally controlled by estimating the phosphoric acid by means of uranium acetate. The neutral salt solutions were made by weighing the desired quantity and then dissolving in distilled water to make up the necessary volume. Carbon dioxide was bubbled through tap water and the resulting fluid titrated against decinormal sodium hydrate in presence of phenolphthalein. As the neutral point to this indicator is reached when all the carbon dioxide is in the form of sodium bicarbonate, the number of c.c. used provides data from which to calculate the concentration of carbon dioxide in solution. Dilutions of this solution in tap water were used for testing the effect of carbon dioxide on the tadpoles. In the tables the strengths are expressed as molecular concentrations.

*The Composition of Liverpool Tap Water*, expressed as parts per 100,000, was as follows:—Total solids, 8 parts, half of which is

organic ; chlorine, 1.1 parts ; hardness, 3.5 (calcium = 1.4 parts) ; phosphates absent.

The reaction of the tap water was compared with distilled water by titrating in the presence of phenol-phthalein and di-methyl-amido-azo-benzole. The quantity of water was large, and, therefore in each case it was necessary to add a certain amount of the standard solution before the ionic concentration of hydrogen or hydroxyl was sufficiently raised to affect the indicator.

1 litre of distilled water to 'phenol-phthalein' needed 1.6 c.c. decinormal alkali

I	"	tap	"	"	"	0.3 c.c.	"	"
I	"	distilled	"	'di-methyl'	"	0.9 c.c.	"	acid
I	"	tap	"	"	"	3.0 c.c.	"	"

From these figures one sees that to both indicators tap water lies to the alkaline side of distilled water and possesses some constituent which very slightly tends to check change of reaction, as shown by adding the number of c.c. necessary to affect the different indicators. Thus for distilled water the limits between which neither indicator is affected are 2.5 c.c. (0.00025 N), whilst for tap water they are 3.3 c.c. (0.00033 N) apart. These values are so small that gas batteries would not show any difference between distilled and tap water, but they are of interest, as showing that with the small quantity of reagent added in the acid and alkali experiments the change of concentration of hydrogen and hydroxyl ions would be slightly less than if the same quantity of solution were added to distilled water.

#### SECTION A. EFFECTS OF ACIDS, ALKALIES, AND SALTS UPON LIFE OF ORGANISM

TABLE I. ALKALIES

Molecular concentration of added chemical in the solution

OBSERVATIONS

(1) Controls

Tap water ... First tadpole died at the end of 46 days, three were dead at the end of 54 days, and at 99 days the remaining three died.

Distilled water First tadpole died at the end of 10 days, two more died 2 days later, and all were dead by the 14th day.

(2) Sodium Hydrate

$0.0003\text{ M}$  }  
 $0.0006\text{ M}$  }  
 $0.0010\text{ M}$  } ... None of these died within 28 days, when the experiment was discontinued.

0.0013 M ... First died in 48 hours, the remainder lived for 20 days when two died, and the three which were left died on the 30th day.  
 0.0017 M ... One died in 22 hours, the remaining five only lived 48 hours.  
 0.0023 M ... First died in 1½ hours, the rest lived only 3½ hours.

(3) *Potassium Hydrate*

0.0003 M ... None of these died within the time of duration of the experiment.  
 0.0006 M ... First died in 17 days, second died in 41 days, three were dead in 57 days, and the remainder died in 66 days.  
 0.0010 M ... First died in 33 days, second died in 42 days, and the remainder died in 57 days.  
 0.0013 M ... First died in 3 hours, three were dead in 15 hours, and all were dead at the end of 46 hours.  
 0.0017 M ... Three died in 3 hours, and the remainder died in 15 hours.

(4) *Ammonium Hydrate*

0.0003 M ... One died in 7 days, two died in 22 days, three were dead in 28 days, and all were dead by the 29th day.  
 0.0006 M ... First died in 16 days, second died in 17 days, four were dead by the end of 20 days, five were dead in 24 days, and the last died on the 27th day.  
 0.0010 M ... Five died in 16 hours, but the last one remained alive for 4 days.  
 0.0013 M ... All died in 55 minutes.

(5) *Calcium Hydrate*

0.0005 M ... First died in 16 days, second in 21 days, third in 23 days, fourth in 25 days, fifth in 34 days, and the last died on the 41st day.  
 0.0007 M ... First died in 20 days, second in 23 days, four were dead in 24 days, fifth died in 27 days, and all were dead in 29 days.  
 0.0008 M ... First died in 18 days, second in 20 days, two more died in 24 days, five were dead in 28 days, and the last died in 46 days.  
 0.0010 M ... First died in 15 hours, second in 94 hours, third in 7 days, fourth in 14 days, and last two in 17 days.  
 0.0013 M ... Four died in 15 hours, and the last two in 94 hours.

(6) *Barium Hydrate*

0.00015 M ... First died in 3 days, two more in 14 days, a fourth in 30 days, and the last two in 39 days.  
 0.00030 M ... First died in 30 hours, two more in 17 days, a fourth in 18 days, and the last two in 30 days.

(7) *Magnesium Hydrate*

A saturated solution of magnesium hydrate was made in distilled water, but the solubility was so slight that no effect was produced on the tadpole.

TABLE II. ACIDS

Molecular concentration of added chemical in the solution	OBSERVATIONS
<i>(1) Hydrochloric Acid</i>	
0.0003 M	... First died in 46 days and the remaining five in 59 days.
0.0006 M	... All dead in 52 hours.
0.0010 M	... Five died in 52 hours but one remained alive for 94 hours.
0.0013 M	... First died in ten hours, a second in 21 hours, and the remaining four in 52 hours.
0.0017 M	... Two died in 4 hours, a third in 5 hours, two more in 7 hours, and the last died in 10 hours.
<i>(2) Sulphuric Acid</i>	
0.0003 M	... First died in 17 days, a second in 23 days, and the remaining four in 24 days.
0.0004 M	... First died in 29 days, a second in 33 days, and the remaining four in 39 days.
0.0005 M	... All died within 15 hours.
0.0006 M	... All died within 15 hours.
<i>(3) Phosphoric Acid</i>	
0.0006 M	... First died in 17 days, a second in 20 days, three more in 20½ days, and the last one died on the 21st day.
0.0010 M	... Five died in 22 hours, and the last one died in 29 hours.
0.0013 M	... In this experiment only four tadpoles were used, and they all died within 15 hours.
0.0020 M	... In this experiment only three tadpoles were used, and they all died within 15 hours.
<i>(4) Acetic Acid</i>	
0.0006 M	... One died in 36 days, and the remaining five in 48 days.
0.0010 M	... First died in 14 days, a second in 17 days, a third in 31 days, and the remaining three in 36 days.
0.0013 M	... Two died in 22 hours, and the remaining four in 45 hours.
0.0015 M	... All died in 39 hours.
0.0017 M	... Five died in 30 hours, and the last one in 45 hours.
0.0033 M	... All died in 16 hours.
<i>(5) Carbonic Acid</i>	
0.034 M	... First three died in 17 days, and the remaining three in 20 days.
0.045 M	... First died in 16 days, a second in 17 days, and the remaining four in 20 days.
0.068 M	... In this experiment only one tadpole was used, and it died in 16 hours.
0.136 M	... In this experiment only one tadpole was used, and it died in 10 minutes.

Molecular concentration of added chemical in the solution

TABLE III. ALKALINE SALTS

Observations

(1) <i>Sodium Bicarbonate</i> ( $\text{NaHCO}_3$ )	
0.0017 M	None of these died within 27 days, when the experiment was discontinued.
0.0023 M	First died in 57 days, and the remaining five on the 75th day.
0.0033 M	Two died in 22 days, a third in 23 days, two more in 25 days, and the last in 28 days.
0.0063 M	First died in 14 days, second in 15 days, third in 18 days, fourth in 21 days, fifth in 22 days, and the last in 28 days.
0.0100 M	In this experiment only one tadpole was used, and it died in 44 hours.
(2) <i>Sodium Carbonate</i> ( $\text{Na}_2\text{CO}_3$ )	
0.0017 M	All lived for 84 days.
0.0023 M	First died in 41 days, a second in 59 days, a third in 61 days, a fourth in 69 days, and the last two on the 82nd day.
0.0033 M	First died in 15 hours, but the remaining five lived for 67 days.
0.0063 M	First died in 19 hours, two more in 45 hours, a fourth in 6 days, a fifth in 14 days, and the last in 23 days.
(3) <i>Di-Sodium Phosphate</i> ( $\text{Na}_2\text{HPO}_4$ )	
0.0007 M	None of these died within 52 days.
0.0011 M	None of these died within 57 days.
0.0015 M	All dead by the 42nd day.
0.0031 M	One dead in 16 days, a second in 20 days, a third in 21 days, two more in 24 days, and the last in 28 days.
0.0232 M	In this experiment only one tadpole was used and it lived for 3 days.

Molecular concentration of added chemical in the solution

TABLE IV. ACID SALTS

Observations

(1) <i>Mono-sodium Phosphate</i> ( $\text{NaH}_2\text{PO}_4$ )	
0.0015 M	None of these died during the continuation of the experiment, which lasted 57 days.
0.0025 M	First two died in 17 days, and a third in 18 days, a fourth in 19 days, a fifth in 21 days, and the last in 23 days.
0.0035 M	In this experiment only one tadpole was used, and it died at the end of 41 hours.
0.0050 M	
0.0373 M	
0.0746 M	

(2) *Sodium bisulphate (NaHSO<sub>4</sub>)*

0.0005 M ... Two died in 26 days, two more in 29 days, and the last two in 39 days.

0.0010 M ... All died in 15 hours.

0.0020 M ... All died in less than 18 hours.

TABLE V. NEUTRAL SALTS

Molecular concentration of added chemical in the solution

## OBSERVATIONS

(1) *Sodium Chloride*

0.100 M ... First died in 4 days, a second in 6 days, a third in 7 days, and the remaining three in 9 days.

0.125 M ... In this experiment only one tadpole was used, and it died in 42 hours.

0.250 M ... In this experiment only one tadpole was used, and it died in 2 hours.

(2) *Potassium Chloride*

0.038 M ... Three died in 9 days, and the remainder in 14 days.

0.044 M ... First died in 6 days, two more in 7 days, another two in 8 days and the last one in 9 days.

0.051 M ... First two died in 5 days, two others in 6 days, and the last two in 7 days.

0.125 M ... In this experiment only one tadpole was used, and it died in 3, hours.

(3) *Ammonium Chloride*

0.033 M ... One died in 12 days, and the remaining five one day later.

0.050 M ... First two died in 21 hours, a third in 24 hours, a fourth in 76 hours, a fifth in 6 days, and the last in 7 days.

0.100 M ... In this experiment only one tadpole was used, and it died in less than 17 hours.

(4) *Calcium Chloride*

0.083 M ... First died in 45 hours, a second in 69 hours, two more in 4 days, and the last two in 7 days.

0.100 M ... Two died in 44 hours, and the remaining four in 92 hours.

0.250 M ... In this experiment only one tadpole was used, and it died in 1 hour and 10 minutes.

(5) *Barium Chloride*

0.0033 M ... All died in 30 hours.

0.0066 M ... All died in 30 hours.

(6) *Magnesium Chloride*

0.033 M ... First died in 12 days, a second in 13 days, and the remaining four in 16 days.

0.050 M ... Two died in 40 hours, and the last four in 46 hours.

0.071 M ... All died in 40 hours.

0.100 M ... All died in 24 hours.

(7) *Sodium Sulphate*

0.033 M ... One died in 10 days, three more in 14 days, a fifth in 15 days, and the last one in 16 days.

0.050 M ... First died in 7 days, a second in 8 days, a third in 10 days, a fourth in 12 days, and the last two in 13 days.

0.066 M ... Five died in 4 days, and the remaining one in 7 days.

(8) *Sodium Acetate*

0.050 M ... First died in 7 days, a second in 15 days, two more in 21 days, a fifth in 40 days, and the last one in 42 days.

0.063 M ... Three died in 7 days, and the remaining three in 14 days.

0.077 M ... First died in 4 days, three more in 7 days, and the last two in 8 days.

0.100 M ... Three died in 5 days, and the other three in 6 days.

(9) *Copper*

A strip of pure copper placed in distilled water killed six tadpoles in less than 15 hours.

(10) *Silver*

Six tadpoles placed in a dilute solution of colloidal silver died in less than 24 hours.

(11) *Platinum*

Six tadpoles placed in a dilute solution of colloidal platinum lived for more than 30 days.

## SUMMARY OF RESULTS GIVEN IN TABLES I TO V

Taking that strength of solution for which at least half the tadpoles used in the experiments died within forty-eight hours, the following may be stated as the lethal concentration of each of the solutions used. In a few cases the values can only be taken as approximate, because at the upper limit the concentration was too suddenly increased, and this was not appreciated until it was too late in the season to obtain a fresh supply of tadpoles :—

TABLE VI

Substance	Lethal concentration		Substance	Lethal concentration	
	Molecular	Ionic <sup>1</sup>		Molecular	
<i>(a) ALKALIES :</i>					
Calcium hydrate ...	0.0013	0.0026 (?)	Sodium bi-carbonate ...	over 0.1000	
Sodium hydrate ...	0.0017	0.0017	Sodium carbonate ...	0.0063	
Potassium hydrate	0.0013	0.0013	Di-sodium phosphate ...	over 0.0465	
Ammonium hydrate	0.0010	0.0010 (?)	<i>(d) ACID SALTS :</i>		
<i>(b) ACIDS :</i>					
Sulphuric acid ...	0.0005	0.0010	Mono-sodium phosphate	over 0.0746	
Hydrochloric acid	0.0013	0.0013	Sodium bi-sulphate ...	under 0.010	
Acetic acid ...	0.0013	0.0013 (?)	<i>(e) NEUTRAL SALTS :</i>		
Phosphoric acid ...	0.0010	0.0010 (?) <sup>2</sup>	Sodium chloride ...	0.1250	
Carbonic acid ...	0.0500	(?)	Potassium chloride ...	under 0.1250	
			Ammonium chloride ...	0.0500	
			Calcium chloride ...	0.1000	
			Magnesium chloride ...	0.0500	
			Sodium sulphate ...	over 0.0660	
			Sodium acetate ...	over 0.1000	

1. These are calculated on the assumption of total dissociation, which would almost be the case at these dilutions, where it is doubtful it is marked (?)

2. Calculated as dissociating to yield,  $H$ ,  $H_2PO_4$ .

An examination of the preceding summarized table shows that the substances tested may be divided roughly into two classes, which exhibit toxic effects at quite different concentrations, viz., (1) the acids and alkalies, and in a less degree certain acid and alkaline salts, which kill at concentrations lying between 0.001 and 0.002 molecular, and (2) the neutral salts which require a concentration at least fifty times as great (0.050 to 0.125 molecular) before acquiring an approximately equal toxic action.

It is probable from this that the action upon the cells in the two sets of cases is different in character, and that in the case of the acids and alkalies the effect is due to a chemical action of the hydrogen or hydroxyl ion respectively upon the protoplasm, while in the case of some of the neutral salts the action is a physical one due to osmotic pressure.

Leaving out of account for the present one or two cases, such as ammonium, barium, and magnesium salts where there seems to exist a special toxic action, it may be pointed out that the concentrations at

which all the alkalies and acids kill lie at the same order of magnitude, and, as in the extremely dilute solutions in which the reactions are occurring the alkalies and acids may be taken as practically completely dissociated, this shows that the action is mainly due to the hydrogen or hydroxyl ion respectively, the other ion being comparatively inert. Further, the figures show that the hydrogen and hydroxyl ion are practically of equal power, which points to a combination between these ions respectively and the proteid of the cell.

Provided the combination between hydrogen or hydroxyl ion and the proteid of the cell were more stable than that between the proteid and the various constituents of the nutrient plasma, with which the cell proteid or protoplasm carries on the metabolism, then such a combination would furnish an explanation of the decrease, and finally the complete stoppage of the metabolic processes and therewith of the life of the cell.

It has been shown by Moore and Roaf<sup>1</sup>, that the action of anaesthetics in limiting the activity of the protoplasm is in all probability due to the formation of chemical compounds or adsorption products between the proteid of the protoplasm and the anaesthetic. So long as the concentration or pressure of the anaesthetic is kept above a certain level these compounds or physical aggregates persist and decrease the power of the protoplasm to undertake the normal processes of metabolism, which must involve a temporary combination or aggregation of tissue proteid and nutrient molecule before the latter can undergo chemical change and oxidation. When the anaesthetic is pushed beyond a certain level of concentration, the metabolic processes are so much hindered that life becomes impossible.

Similarly, acid intoxication leads to unconsciousness or coma, because the acid products reaching the cells form more stable compounds with the proteid of the protoplasm than do the normal nutrient materials, and so lead as the concentration is increased to stoppage of metabolism and of the supply of energy necessary for carrying on the vital processes of the cell.

1. *Roy. Soc. Proc.*, Vol. LXXIII, 1904, p. 382-412, and B, Vol. LXXVII, 1905, p. 86-102.

There is little doubt that the low concentrations of acid or alkali which have been shown to lead to death in the above experiments produce their effects in a similar fashion by forming relatively stable compounds or aggregations with the cell protoplasm, which are formed even at very low ionic concentrations.

It has been pointed out by Moore<sup>1</sup> that the protoplasm of the cell must be regarded as in labile equilibrium with the nutrient materials which are supplied to it so that it can enter and pass out of combination, physical or chemical, with the various constituents. The point of equilibrium at each instant depends on the concentration, or osmotic pressure, of each constituent in the cell for the time being. In order that this power of the cell may remain in action it is necessary that there shall not be present in the cell (at any rate beyond given concentrations) any constituents which enter into stable combination with the protoplasm, otherwise combination with such constituents will occur to the exclusion of the labile metabolic combinations.

An example may make the above view clearer. Haemoglobin enters into labile combination with oxygen to form oxy-haemoglobin, and according to the osmotic pressure or concentration of the oxygen in the plasma, the point of equilibrium shifts, and combination or dissociation occurs. Similarly, carbon monoxide combines with haemoglobin to form carboxyhaemoglobin, but the combination is much more stable, and the equilibrium point lies much closer to complete formation of carboxyhaemoglobin at much lower pressures of carbon monoxide. Now if haemoglobin be subjected *simultaneously* to the action of given osmotic pressures of oxygen and carbon-monoxide in the plasma, there will be at any instant given proportions of the haemoglobin in combination with oxygen and carbon-monoxide respectively, the amount depending upon the two osmotic pressures and the relative stabilities of the two compounds. On account of the higher stability of the carbon-monoxide compound, a comparatively low concentration of carbon-monoxide in the plasma will enormously

1. *In Recent Advances in Physiology and Bio-Chemistry*, edited by Leonard Hill, Arnold, 1906, pp. 148-159.

diminish the amount of oxy-haemoglobin, and correspondingly diminish the oxygen carrying power of the blood. This is the explanation of carbon-monoxide poisoning.

Exactly similar reasoning holds in the case of the protoplasm of the living cell, and the materials in the nutrient medium with which it reacts ; the nutrient proteid, carbohydrate, and fat on the one hand, and toxic materials, such as anaesthetics, hydrogen, or hydroxyl ions on the other. The toxic materials limit and finally stop the reaction between the cell and its nutrient substances, by forming more stable compounds with the protoplasm of the cell. For each toxic substance the equilibrium point between nutrient matter and toxic substance in their reaction with the protoplasm will depend upon relative concentrations or osmotic pressures, and the degree for each of stability of combination<sup>1</sup>. Since the concentration of the nutrient materials may approximately be taken as constant, it follows that for each toxic substance there is a given concentration at which it becomes fatal to the life of the cell, and the lower the fatal concentration the higher accordingly must be the stability of the combination between it and the cell protoplasm. So that the figures given above in the table might be regarded as coefficients of the affinity of each substance for combining with cell protoplasm<sup>2</sup>.

Moore and Roaf<sup>3</sup> have given experimental evidence along different lines for the existence of such combination between proteid and anaesthetic in the case of a large number of anaesthetics.

The evidence in the literature of combinations between proteid and alkali or acid is too well known to require detailed description.

Barrett<sup>4</sup> in the case of *Paramoecium*, has brought forward evidence from measurements of conductivity and electrical potential that added acids or alkalies pass into combination with the tissue of the organisms which is convincing, although, as shown in a succeeding section, part of the observed changes may be ascribed to excretory substances thrown out by the organisms.

1. Shown by the constant (*k*) of the equilibrium equation.

2. The question whether the combination is due to a chemical compound, or to a physical adsorption or aggregation may be left open, and is not vital to the issue.

3. *Loc. cit.*

4. *Zeit. f. Allgemeine Physiol.*, Vol. IV, 1904, p. 438, and Vol. V, 1905, p. 10 ; also, *British Medical Journal*, 1906, Vol. I, p. 129.

On these grounds the conclusion may be drawn that various substances or ions in solution which affect cells do so by combining with the cell substance to form compounds or aggregates of varying stability which interfere with the play of activity between the cell substance and its normal nutrient constituents ; and that the effect is proportionate (1) to the concentration of the toxic substance, and (2) to its combining power with the cell substance.

Secondly, in the case of the alkalies and acids the main factor is the concentration of the hydrogen or hydroxyl ion, and the other ion has little effect.

The neutral salts only commence to show injurious or lethal effects at a much higher concentration where the osmotic pressure begins to exceed that of the organism, and as the effects are bound up with changes in volume of the organism which always precede them the phenomena are discussed in a separate section.

#### SECTION B. EFFECTS OF NEUTRAL SALTS UPON THE VOLUME OF THE ORGANISM

One of the most striking results in the whole research was the wonderful diminution in volume of such a complicated organism as the tadpole, which was compatible with life, and the fact that marked diminution in volume was obtained while the concentration of the salt in the surrounding fluid was still obviously much below that of the tissue fluids of the organism itself.

The latter fact is of high interest in view of the usually accepted view that the living cell does not shrink in volume until the osmotic pressure of the surrounding fluid is equal to that of the cell contents.

This, in fact, has hitherto formed an axiom in measuring the osmotic pressure of living cells, such as the blood corpuscles, frog-muscle, etc. It has been assumed that, if a living cell neither shrinks nor swells when placed in a given saline medium, that then the said medium is isotonic, or of equal osmotic pressure, with the cell contents.

The results of our experiments with living tadpoles, show clearly however, that this position must be reviewed, and that certain animal

cells and tissues may decrease in size long before the osmotic pressure without is equal to the osmotic pressure within.

In the case of the tadpole, and probably in the case of other living organisms, the criterion of unaltered volume of the cell does not appear to be equality of osmotic pressure within and without, but rather a constant and fixed difference of pressure within and without, to which the cell appears to accommodate itself, and which is normal to the organism.

Further, the particular organism with which we have worked appears to be able to live with widely different osmotic pressures in the external medium, and to be able to accommodate itself to those pressures, still preserving, by shrinkage in volume, a positive difference between the internal and external pressures.

We have seen tadpoles living and *active* for days, although shrunken to approximately one-third of their normal size, in solutions of different neutral salines, which even then only began to approximate in osmotic pressure to the normal osmotic pressure of the tissues of the adult frog.

It is remarkable that the concentrations of saline at which death of the tadpoles began to occur fairly rapidly, coincided roughly with the normal osmotic pressure of the adult organism. Thus, the lethal concentration of sodium chloride and potassium chloride for tadpoles was nearly the same, approximately 0.125 M, corresponding to a depression of freezing point of 0.4332° C.,<sup>1</sup> while the osmotic concentration of frogs' blood is 0.1093 M NaCl<sup>2</sup> corresponding to a depression of freezing point of 0.3789.<sup>1</sup>

This result is of interest as giving an indication of how the living cells in this particular organism react to increased osmotic pressure in their external medium. The cells do not remain of constant volume, as the osmotic pressure of the external medium is run up from that of tap water to the concentration of the internal medium or above it; but there is a change of volume throughout, although the osmotic pressure in the external medium is a long way

1. Calculated from data given by HAMBURGER, *Osmotischer Druck*, Wiesbaden, 1902, Vol I, p. 83.

2. *Ibid.* p. 181.

below that of the tissue fluids. By the time the osmotic pressure in the external medium is approximately that of the internal medium the tadpole may have shrunk to approximately one-third of its normal volume.

Finally, the death limit lies, especially when the salt used for raising the osmotic pressure in the outer medium is foreign to that chiefly constituting the medium of the cell, just above that of the internal medium, assuming that the osmotic pressure in the tissues of the tadpole is approximately that of the adult frog.

It hence appears clear that the living cells in the tadpole possess a regulatory mechanism which tends to preserve a constant difference in osmotic pressure between the external and internal media, as shown by the shrinkage long before the external medium possess the osmotic pressure of the internal medium, and that it is only after this mechanism has been overcome by the osmotic pressure of the external medium becoming higher than that of the internal medium that any foreign ions in the external medium enter the cell in appreciable quantity, and lead to the death of the cell and of the organism.

Thus, comparing the action of sodium chloride and potassium chloride, as shown in Table V, it is not until 0.1 M concentration is attained that either salt begins to show marked poisonous effects although the tadpoles in both solutions have at this concentration shrunk to mere pygmies of their former selves, but at 0.125 M concentration the potassium chloride kills in three hours, while the sodium chloride takes forty-two hours to kill.

Now, it is well known that the potassium ion once it enters the cell in much more than normal concentration rapidly kills, hence it is evident that it is only when the concentration or osmotic pressure without is equal to that within that the potassium ion begins to enter rapidly, and the potassium ion, being more poisonous than the sodium ion, leads more rapidly to a lethal result.

It is probable that the conditions in all living cells are not the same, the blood cells exist in a fluid, the plasma, of constant composition and osmotic pressure, and have developed under conditions in which the osmotic pressures within and without are equal; but in

other cells, as shown by these experiments, there is no necessity for equality of osmotic pressure within and without, but rather there is a mechanism for preserving a constant difference of osmotic pressure between the internal and external media of the cell.

Hence, while it may be safe to take the constancy of volume as a criterion of equality of osmotic pressure in the case of the blood cells, it is quite otherwise in the case of other cells. Even in the case of mammalian tissues it probably requires further investigation before assuming that a solution, in which the tissue neither gains nor loses weight, is iso-osmotic with the tissue fluids.

### SECTION C

#### EFFECT OF WEAKER SOLUTIONS ON RATE OF DEVELOPMENT

TABLE VII

(a) WEIGHTS

Nature of added chemical	Molecular concentration of added chemical in the solution	Average weight of each tadpole at end of 27 days	Nature of added chemical	Molecular concentration of added chemical in the solution	Average weight of each tadpole at end of 27 days
Tap water	—	0.107 g	Sodium carbonate	0.0017 M	0.098 g
Sodium hydrate	0.0003 M	0.123 g	Di-sodium phosphate	0.0023 M	0.073 g
	0.0006 M	0.098 g		0.0033 M	0.072 g
	0.0010 M	0.093 g		0.0007 M	0.148 g
	0.0013 M	0.116 g*		0.0011 M	0.130 g
Potassium hydrate	0.0003 M	0.128 g	Mono-sodium phosphate	0.0015 M	0.127 g
	0.0006 M	0.078 g		0.0031 M	0.117 g
	0.0010 M	0.090 g		0.0047 M	0.125 g
Sodium bicarbonate	0.0017 M	0.100 g	Hydrochloric acid	0.0015 M	0.147 g
	0.0023 M	0.108 g		0.0025 M	0.137 g
	0.0033 M	0.100 g		0.0035 M	0.122 g
	0.0063 M	0.103 g		0.0050 M	0.113 g

\* In this only three remained alive, and as probably the weakest died first, the weight of the remaining ones would be above the average.

## (6) STAGES OF DEVELOPMENT

Nature of added chemical	Molecular concentration of added chemical in the solution	OBSERVATIONS
Sodium carbonate	0.0017 M	One reached the stage, when four legs appear, in 40 days; a second in 41 days. One tail almost gone in 42 days.
	0.0023 M	One reached four-legged stage in 59 days.
Sodium bicarbonate	0.0063 M	One reached four-legged stage in 45 days.
Di-sodium phosphate	0.0007 M	Four reached four-legged stage in 40 days, and a fifth in 41 days. Three tails had almost disappeared in 42 days, and five in 45 days.
	0.011 M	One reached four-legged stage in 40 days, and a second in 41 days. One tail had almost disappeared in 40 days.
	0.0015 M	One reached four-legged stage in 40 days, a second in 42 days. One tail almost gone in 42 days.
	0.0031 M	Two reached four-legged stage in 42 days.
	0.0047 M	One reached four-legged stage in 40 days, a second in 41 days, and a third in 42 days.
Mono-sodium phosphate	0.0015 M	Three reached four-legged stage in 40 days, and a fourth in 41 days.
	0.0025 M	One reached four-legged stage in 40 days, and a second in 46 days.
	0.0035 M	One reached four-legged stage in 42 days, a second in 45 days, and a third in 46 days.
	0.0050 M	One reached four-legged stage in 40 days, and a second in 41 days.
Hydrochloric Acid	0.0003 M	One reached four-legged stage in 41 days, and a second in 42 days.

From the table it is seen that the rate of growth is greater in the weakest solution used of sodium hydrate and potassium hydrate (0.0093 M) than in tap water. There is no increase with the alkaline carbonate. Both phosphates cause an increase in all strengths up to about 0.05 M. The tadpoles only lived in the least concentrated solution of hydrochloric acid used (0.0003 M), but having survived this concentration, the organisms developed more rapidly than in tap water.

The results obtained in the case of mono-sodium phosphate and hydrochloric acid differ from those obtained by Moore, Roaf, and Whitley<sup>1</sup> in observations upon *Echinus esculentus*, but it must be remembered that we are here dealing with a much more complicated organism possessing a specialized excretory mechanism, and hence there is probably a neutralizing action on the part of the organism. The great increase in growth with the phosphates points to a special stimulating action of these salts upon cell-division and growth, the acid phosphate absorbed being probably converted into alkaline phosphate by the activity of the ammonia formed by the organism (see Section D).

#### SECTION D. EFFECTS OF EXCRETORY PRODUCTS FROM THE ORGANISMS UPON THE EXTERNAL MEDIUM

In considering the effects of ions in varying concentration, present in the external medium, upon the living organism, as we have been doing in the preceding sections, it must not be forgotten that there is also to be considered the action of the organism, or its excretory products, upon the external medium. Such action may easily alter the ionic concentrations in the external medium, unless the volume of the external medium is kept large, compared to the volume of the organism, and the amount of their excreta, and the water be regularly changed.

Thus, we have seen in a preceding section, that the effects of the ions are due to their forming compounds or aggregates, of varying degree of stability, with the cell protoplasm, until an equilibrium point is attained in each individual case. As a result of this aggregation, the different ions and dissolved substances will be taken up, each in definite amount, by the protoplasm from the solution, and it is only when the volume of solution compared to volume of organism is large that such uptake will not appreciably effect the concentrations of dissolved substances in the external medium. Also, the excretion of bodies by the action of the cell's metabolism may neutralize added reagents in the external medium, and cause changes in ionic

1. *Loc. cit.*

concentrations. This factor may be guarded against by frequent changing of the external medium.

By preserving a large volume of fluid, compared to the volume of the tadpoles used, and by frequent changing, we have endeavoured to secure that the concentrations of the salts and ions tested by us should remain throughout each experiment close to the concentrations initially made up.

We thought it well, however, to investigate the amount of change produced by tadpoles in media similar to those we had experimented with in the preceding sections. For this purpose, one litre of solution was used in each experiment, and in one of two similar vessels, 24 tadpoles were placed, while the other was used as a control, and had no tadpoles added. The media tested in this way were 0.0005 M hydrochloric acid, and 0.0005 M sodium hydrate, and the duration of the experiment was in each case twenty-four hours. At the end of this period, the 24 tadpoles were removed, and the following determinations made :—(1) The change in reaction to di-methyl-amido-azo-benzol, and to phenol-phthalëin, as contrasted with the controls, by titration with deci-normal alkali or acid ; (2) the amount of ammonia formed by the tadpoles, estimated by using Nessler's reagent ; (3) the amount of carbon-dioxide formed and going to neutralize caustic alkali, and so reduce hydroxyl ion concentration in the case of the alkaline medium ; this was estimated by passing a current of air free from  $\text{CO}_2$  through the acidified solution, drying by pumice impregnated with dried copper sulphate, absorbing by soda lime and weighing. The results are shown in the following table :—

Reagent added to medium	Weight of tadpoles	Change of reaction in c.c. of deci-normal solution to (a) di-methyl (b) phenol-phthalëin	Ammonia formed in c.c. of deci-normal solution	Carbon-dioxide formed in c.c. of deci-normal solution
0.0005 M hydrochloric acid	2.38 grams	1.2 c.c. less acid than control	0.59 c.c.	—
0.0005 M sodium hydrate	2.26 grams	0.8 c.c. more alkaline than control	0.54 c.c.	0.4 c.c.

NOTES.—Since di-methyl is practically unaffected by carbon dioxide, the loss of acid in the acid medium must be made up of combinations of organisms with acid and ammonia. The total loss being 1.2 c.c. and the amount of ammonia excreted 0.59 c.c., the amount of acid lost in the organisms by combination, probably with protoplasm, is  $1.2 - 0.59 \text{ c.c.} = 0.61 \text{ c.c.}$  In the experiment with sodium hydrate the gain in alkalinity is 0.8 c.c., and the amount of ammonia is only 0.54 c.c., and since there must have been some amount taken up by the tadpoles, it follows that there must have been an excretion of other basic bodies by the tadpoles.

The results on such small quantities cannot possess quantitative accuracy, but show that the ionic concentrations in the external medium undergo slight changes, due in part to absorption by the organisms, and in part to excretion.

On account of the second of these two factors it is obviously impossible to follow accurately by exact physical methods, such as determinations of electrical conductivity and potential the proportions in which the organism takes up or combines with ions present in the external medium, as has been attempted recently by Barrett.<sup>1</sup>

Interesting and valuable as such experimental observations undoubtedly are, they do not give quantitatively the amount of combination of added ions with the protoplasm, because the excretory products given off at the same time by the organisms also alter the electrical conductivity and potential to hydrogen electrodes of the medium, and since it is difficult or impossible accurately to discount this effect, the change in conductivity or potential do not afford a measure of combination between protoplasm and the added ions in the external medium.

The combinations between ions and protoplasm, and the degree of stability of these is, hence, better measured by the physiological effects.

#### SUMMARY AND CONCLUSIONS

1. For the organism experimented with, the ions which possess the most powerful action are the hydrogen and the hydroxyl ion, which kill in concentrations of 0.001 to 0.002 M. The degree of toxicity is of the same order of magnitude for these two ions. The most probable explanation of this result is that these ions form more stable compounds or aggregations with the protoplasm than do the ions of the neutral salts. In the case of salts, which, by hydrolysis or dissociation in solution, alter the concentrations in hydrogen or hydroxyl ions in the medium, such as the carbonates and phosphates of the metals of the alkali group, and acid sodium sulphate, the degree of toxicity is roughly proportional to the concentration in hydrogen or

1. *Loc. Cit.*

hydroxyl ion. Thus, sodium bi-carbonate requires the same concentration as a neutral salt (over 0.1000 M) before it kills ; sodium carbonate corresponding to its higher concentration in hydroxyl ions, kills at 0.0063 M ; while sodium bi-sulphate, which is almost completely dissociated, corresponding to its high concentration in hydrogen ions, kills at less than 0.0010 M.

2. Neutral salts, such as sodium chloride, potassium chloride, and calcium chloride, when present along with traces of other ions, as in tap water, do not kill until the osmotic pressure rises above that of the fluids of the organism. But, when the concentration becomes hypertonic, compared with that of the body fluid of the organism, then even these salts rapidly commence to be fatal. So that there is a long range of concentrations where such salts, under our conditions of experimentation, are comparatively harmless, and then a short range in which they become rapidly fatal. The length of this short range varies with the ions of the neutral salt, the potassium ion, for example, after isotonicity has been passed, being much more rapidly fatal than the sodium ion. This we take as an indication that the ions of the neutral salt used do not rapidly increase in concentration within the cells of the organism until the isotonic point has been passed.

3. The organism does not remain constant in volume until the surrounding medium has become isotonic with the internal medium, but diminishes constantly in volume as the concentration of the outer medium is increased, although this is still very hypotonic. In the neighbourhood of the isotonic point the volume may be only about one-third of the normal volume. Hence, the fact of unchanged volume when living cells are placed in different media cannot always be taken as an indication of isotonism between such solutions and the cell fluids.

It appears from this that the organism we have used tends to preserve not equal osmotic pressures of inner and outer media, but possesses a regulating mechanism which preserves a higher pressure within than without up to the point at which the outer pressure approximately equals the normal internal pressure ; beyond this point the mechanism is upset, and the lethal effects rapidly appear.

Some neutral salts contain ions with specific poisonous effects upon protoplasm, and kill on this account when the outer medium is still very hypotonic, such as salts of all the heavy metals, barium salts, and ammonium salts.

4. A colloidal solution of platinum, as concentrated as we were able to prepare it, did not appear to have the slightest effect upon tadpoles, while a very dilute colloidal silver solution killed in less than twenty-four hours. The latter effect might, however, be due to a trace of silver in solution.

5. At much lower concentrations than those which give lethal results, certain ions possess a favouring action upon growth. Of those salts which we have tested, this action is most pronounced in the case of the phosphates. A less amount of increase is seen with potassium and sodium hydrates, and also with the lowest concentration of hydrochloric acid used ( $0.0003\text{ M}$ ), the tadpoles perishing with all the higher concentrations.

6. The excretory products given out by the organisms render it experimentally impossible to determine, by physical methods, the exact amount of combination between the organisms and the ions of the added solutions in the external medium.

In conclusion, we wish to express our thanks to Professor B. Moore for his valuable assistance and constant advice during the course of these experiments.

## OBSERVATIONS ON FEHLING'S TEST FOR DEXTROSE IN URINE

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### INTERPRETATION OF CERTAIN RESULTS OBTAINED WITH FEHLING'S TEST

For the detection of a comparatively large amount of sugar, such as is generally in evidence in ordinary diabetic urine, Fehling's test gives very definite results. When applied, however, in the usual way its indications are so often ambiguous as to render it of little service for the detection of small amounts of sugar. Its reactions, moreover, are not constant, for an amount of sugar sufficient to give a fairly distinct reduction and precipitation of cuprous oxide or hydrate in a certain urine, may in another sample give little or no indication of its presence on the most careful application of the test as ordinarily applied. On the other hand, such a modified reaction may be obtained as to render the interpretation difficult or impossible, one observer, perhaps, thinking that a trace of sugar is present, another crediting excess of some 'interfering' substances with the production of the change. Again, it would seem that every urine is capable of giving a well-marked reaction with Fehling's solution if equal parts of the solution be mixed with the urine and boiled for some time ; the reaction usually consists in the separation of a well-marked precipitate, generally of a yellowish appearance—probably cuprous hydrate, or a mixture of the latter with the red cuprous oxide. The time required for the production of this reaction varies greatly in different urines, but in general, boiling for about three to four minutes is sufficient ; sometimes less than two minutes is required, while in other cases up to eight minutes may be necessary. Since Fehling's solution mixed with water gives no yellow or red precipitate

however long it is subjected to heat, it is obvious that the phenomenon is not the result of prolonged boiling on Fehling's solution, but depends directly on the urine. This reduction on prolonged boiling with normal urine suggests the necessity for limiting the amount of boiling when testing for sugar in doubtful cases.<sup>1</sup>

If the application of the test be modified, more accurate results can certainly be obtained with comparatively small amounts of sugar, but this necessitates more time and labour than the majority of clinicians are prepared to give, and at the same time renders the test somewhat complex in application. Of these modified methods that advocated by Allen<sup>2</sup> is perhaps the best; the greater accuracy obtained by this method depends on the utilization of the copper sulphate part of Fehling's solution for the precipitation of the majority of interfering substances present in the urine, the addition of a trace of acetic acid enhancing the result; uric acid, xanthine, hypoxanthine, and albumin (if present) are thrown down *in toto*, but phosphates and kreatinin only partially. After separation of these substances by filtration, the greenish-blue liquid obtained is boiled with the alkaline part of Fehling's solution and reduction takes places more or less quickly in proportion to the relative amounts of dextrose present. In the presence of from 0·2 to 0·3 per cent. of sugar separation of cuprous oxide occurs before the boiling point is reached, but with smaller quantities deposition takes place during the cooling of the solution, which first becomes greenish, ultimately giving a yellow precipitate. In applying the above modification, however, it was found that small amounts of sugar could not always be detected, and that some interfering substances seemed in many cases to be still present. Normal urines to which small amounts of chemically pure dextrose had been added often failed to respond even to Allen's modification, and different urines seemed to react differently, for while it was often easy to detect a certain amount in one urine, it was sometimes impossible to recognise dextrose in another containing

1. A solution of kr. atinin in water containing, approximately, the same percentage of kreatinin as ordinary urine gives, on prolonged boiling, quite a similar reduction to that obtained from urine; it would thus seem that the reduction observed on boiling normal urine for some considerable time is due to kreatinin.

2. Allen, *Chemistr. of Urine*, 1895, pp. 62-63.

twice the amount of sugar added to the first. In the absence of interfering substances, however, Fehling's solution is one of the most delicate indicators of sugar we possess ; if distilled water be used as a solvent such minute traces of sugar as 1 in 120,000 can be distinctly demonstrated. The following table<sup>1</sup> shows the delicacy of the more common tests for sugar in aqueous solution :—

Fehling's test	= 0.0008	per cent.
Trommer's test	= 0.0025	"
Nylander's test	= 0.025	"
Fermentation test	= 1.05	"
Phenyl-Hydrazin test	= 0.025-0.05	"
Polarimetric test	= 0.025-0.05	"

No normal urine, however, will give the slightest indication of the presence of sugar on the addition of many times 0.0008 per cent. of dextrose ; this at once leads to the conclusion that urine must contain something which prevents sugar, when present in small amount, giving any appreciable reaction with Fehling's solution. Pavy explained this phenomenon, on the assumption that the action of the alkaline constituents of Fehling's solution on the nitrogenous constituents of the urine, might generate free ammonia in quantity sufficient to hold the reduced cuprous oxide in solution, and so form no precipitate. In order to test this, various artificial urines were made up, each containing the chief nitrogenous constituents of urine<sup>2</sup> in slightly varying proportions, together with the ordinary salts ; to these artificial urines, traces of sugar from 0.0008 per cent. upwards, were added ; on boiling these with Fehling's solution, however, there was a very distinct reduction in every case, and it was found that even the uric acid present was quite sufficient to give a fairly distinct reduction without the addition of any sugar ; indeed, it was obvious from these experiments that normal urine must contain much more uric acid than is necessary to reduce Fehling's solution in ordinary aqueous solution in the absence of interfering substances. Moreover, the addition of free ammonia in quantities greater than would likely

1. Simon's *Clinical Diagnosis*, 1904, p. 522.

2. Except kreatinin.

be present in urine, either free or loosely combined, did not seem to affect the delicacy of the test to any appreciable extent. Were the reaction due to ammonia present in loose combination, boiling the urine with the alkaline part of Fehling's solution for a comparatively short time, should be sufficient to remove it; urines, however, to which small amounts of dextrose have been added previously, do not give any more indication of the presence of sugar after boiling than before.

An estimation of the amount of ammonia given off from an artificial urine, which showed very distinctly the presence of small traces of sugar with Fehling's solution, and from a normal urine which gave no indication of a relatively much greater amount of sugar, resulted in a somewhat greater yield of ammonia having been obtained from the artificial urine.

All this is contrary to the idea that ammonia is the substance that prevents small amounts of sugar from showing with Fehling's solution in normal urine; while it may have a certain effect in some cases, it is ordinarily evolved in too small an amount to markedly interfere with the reaction. As the result of many experiments, it was found that the substance normally present in urine which tends to prevent small quantities of sugar reacting with Fehling's solution in the ordinary way and interferes most with the test is kreatinin. As Allen's modification of this test does not get rid of all the kreatinin present, it is not quite trustworthy for small amounts of sugar.

#### REACTIONS OF KREATININ WITH FEHLING'S SOLUTION AND THE MANNER IN WHICH IT MODIFIES THE REACTION OF DEXTROSE WITH THE REAGENT

Despite the amount that has been written in regard to Fehling's test for dextrose, I have been quite unable to find any definite statement of the exact manner in which kreatinin modifies the results of this test. From the statements made in the ordinary text books that kreatinin is an 'interfering' or 'reducing' agent, it might be inferred that a strong aqueous solution of kreatinin when heated with Fehling's

solution would give a reduction and precipitate which might be mistaken for sugar. This, however, is not so. Kreatinin does reduce Fehling's solution in the sense of decolourizing the blue solution, but a pure sample gives no precipitate unless the boiling process is very much prolonged. If a small quantity of kreatinin, such as might be present in urine, be dissolved in some water and Fehling's solution added no change whatever results, unless on prolonged boiling ; the addition of larger amounts reduces the blue colour till ultimately a clear solution is obtained ; this clear solution becomes yellow if more is added, but no precipitate is obtained.

In the specimens' used in this investigation, the following results were obtained on boiling solutions of kreatinin with an equal volume of Fehling's solution :—

1 mgr. kreatinin in 1 c.c. H <sub>2</sub> O	=	No change whatever even on somewhat prolonged boiling ; no change after cooling.
3 mgr. " " 1 c.c. "	=	No change on boiling ; the colour seemed to be slightly reduced on cooling, but this is hardly apparent.
5 mgr. " " 1 c.c. "	=	Partially decolourized on boiling ; on cooling distinct reduction of colour, but still fairly blue.
9 mgr. " " 1 c.c. "	=	Distinct decolourization on boiling ; after cooling showed almost clear solution.
15 mgr. " " 1 c.c. "	=	Decolourized on boiling, giving yellow solution distinctly yellow on cooling ; no ppt.

G. S. Johnson<sup>2</sup> found that 0.0284 grm. of sarcous kreatinin reduced 40 c.c. of Pavy-Fehling solution ; 40 c.c. of Pavy-Fehling solution being equivalent to 4 c.c. of ordinary Fehling's solution it follows that 1 c.c. of Fehling's solution would be reduced by  $\frac{0.0284}{4}$  gram = 7.1 mgr. kreatinin. This corresponds approximately with results given above. Since the amount of kreatinin present in urine is variously estimated at an average of from .5 to 2 grammes per diem. (= 0.33 - 1.33 mgr. per c.c. taking 1,500 c.c. as normal amount of urine per diem) it cannot decolourize the ordinary Fehling's solution to any appreciable extent

1. Samples were obtained from Merck and from Grüber ; kreatinin was also prepared from the urine by Johnson's method.

2. *Proceedings Royal Society, London, Vol. L, 1891.* He put the reducing power of urinary kreatinin somewhat higher.

in virtue of its own direct reducing power even when present in excess, such as 4 grammes per diem or so ; in urine, moreover, to which a sufficient amount of kreatinin has been added to give a reaction with Fehling's solution the result is somewhat different from that obtained in aqueous solution ; in urine as a rule it tends to give a light greenish coloured fluid rather than a clear liquid. This direct reducing power possessed by kreatinin is therefore of comparatively little importance in regard to its interference with Fehling's test unless the kreatinin be present to a very abnormal extent.

Kreatinin, however, markedly interferes with the results of Fehling's reaction in other ways.

1. By its power of preventing small quantities of dextrose from giving a reaction with Fehling's solution.
2. By its power of modifying and masking the reaction of dextrose when present in larger amounts.

With regard to its power of masking the presence of small amounts of sugar, it is probable that the cuprous hydrate or oxide is held in solution by kreatinin, and so does not become apparent in the ordinary way. Quantities of sugar which, in aqueous solution, give quite a marked reaction with Fehling's test, give no apparent reaction whatsoever—no decolourization, etc.—after the addition of a small amount of kreatinin ; if the amount of sugar present is small, a comparatively small amount of kreatinin is quite sufficient to prevent any reaction ; if on the other hand, the solution contains a larger amount of sugar than can be prevented from showing by the amount of kreatinin present, a modified and ambiguous reaction results. An aqueous solution containing about '08 per cent. of sugar, gives a very strong reaction with Fehling's test ; if 1 c.c. of this solution be mixed with an equal amount of Fehling's solution, and from 2-3 mgr. kreatinin added, no change whatever is observed, even after boiling for some time ; if somewhat less than 2 mgr. kreatinin be added, and the mixture boiled, no marked change is seen for some time ; after cooling, however, the liquid usually undergoes a change—first it becomes greenish-blue, then greenish, and finally assumes a greenish-yellow opalescent appearance—the reaction is much modified and

changed. Stronger solutions of sugar require correspondingly larger amounts of kreatinin to prevent the reaction, but considerable quantities of sugar can be prevented from giving any precipitate by means of kreatinin, only in this case the blue solution is more or less modified in colour. Since, according to Voit, kreatinin may be present in normal urine up to about 3 mgr. per c.c., it is obvious that pathological amounts of sugar might be obscured or masked by this substance, especially in concentrated urines. Kreatinin, as stated, when present in sufficient amount to give a slight decolourization, tends to give, not a clear, but a greenish solution : this, however, is due to the modifying influence of dextrose or some other reducing substance present, seeing it never gives this in aqueous solution, and depends on the relative amounts of kreatinin and reducing substance present, which may or may not be dextrose. In these urines, however, it is often found, that subjection of the urine to yeast fermentation results in the elimination of this power of changing the blue Fehling's solution to green ; this points to traces of sugar being the cause of the change ; in fact, it would seem that in the majority of cases in which the Fehling's solution is changed to a dense green colour, dextrose, and not other reducing substances, constitutes the chief factor in the change. Repeated examination of a urine shewing this phenomenon will sometimes result in the detection of an unmistakable quantity of sugar, indicating that such urines should be regarded with suspicion. On the other hand, a urine, poor in kreatinin, and rich in some reducing substance, such as uric acid, might give the same reaction, though, for reasons mentioned below, it would probably not cause confusion. Glycuronic acid, if present, might give it however.

The power possessed by kreatinin of modifying in quite a different manner the reaction of dextrose with Fehling's solution is perhaps its most important characteristic from the clinical point of view at any rate. It is sometimes found that a urine will for some time give no indication of any decolourization or precipitate with Fehling's test, or it may give a slight greenish colouration ; this, however, may not happen till it stands for some time after heating ; soon, however, this green colour becomes more marked, till ultimately

the liquid becomes of a dirty milky green, ultimately assuming a very characteristic greenish-yellow opalescent appearance. There is generally no precipitate. This reaction is often mentioned in works on urine examination, but there seems to be no explanation of its cause or significance. Simon<sup>1</sup> in his book on *Clinical Diagnosis* states that not infrequently when applying Fehling's test it will be observed that upon standing when no precipitate has occurred previously the blue colour of the mixture changes to emerald green, while the solution at the same time becomes turbid, but that such a phenomenon should not be referred to the presence of sugar, as it is, in all probability, due to the action of other reducing substances. Allen,<sup>2</sup> on the other hand, while admitting that a urine giving a reaction similar to that described above may contain sugar, explains the phenomenon on the assumption that some of the reducing substances of urine, such as uric acid, hippuric acid, hypoxanthine, glycuronic acid, or kreatinin may be preventing the normal reactions of sugar.

The substance, however, which causes this modified action is kreatinin. The other reducing substances which may be present in the urine to a greater or less extent never seem to interfere with the ordinary sugar reaction in the manner indicated. If the amount of kreatinin present is barely sufficient to entirely prevent a reaction with Fehling's solution, there is often no result on boiling, but, soon afterwards, the greenish colouration appears, and the liquid may become gradually an opalescent, dirty, greenish fluid; if the amount of kreatinin is small, or the sugar relatively large, the reaction occurs in a short time; the reverse also holds good, and sometimes the reaction is postponed for six minutes and even longer. If to a solution acting as above a very slight amount of kreatinin be added, the reaction is postponed still longer, or may hardly be in evidence at all. The following results were obtained with solutions of dextrose in distilled water, treated with equal amounts of Fehling's solution:—

1 c.c. of '08 per cent. sugar solution } No change even after standing for  $\frac{1}{2}$ -hour after  
with  $2\frac{1}{2}$  mgr. kreatinin      ... } boiling.

1. Simon, *Loc. cit.*, p. 518.  
2. Allen, *Loc. cit.*, p. 61.

1 c.c. of '1 per cent. sugar solution	No change on heating ; some time after cooling, with 2 mgr. kreatinin ...	dirty yellowish opalescence.
1 c.c. of '15 per cent. sugar solution		
with 3½ mgr. kreatinin ...	Slight greenish-yellow decolourization of fluid, but no ppt. even on cooling.	
1 c.c. of '4 per cent. sugar solution		
with 3 mgr. kreatinin ...	Decolourized on boiling ; greenish-yellow ; after cooling, very distinct, dirty yellow, opales- cence in fluid.	

To give this modified reaction two substances are necessary :

(a) Kreatinin.

(b) Substances reducing cupric oxide, and capable of giving a precipitate in the ordinary way with Fehling's solution.

Of class *b*, the chief substances usually present in the urine are dextrose and uric acid, and in rare instances, glycuronic acid. Neither kreatinin nor any of these substances will give the reaction alone, but any one of class *b* will give it when present in certain quantities in a liquid having a certain proportion of kreatinin. Uric acid, however, will only act when present in much larger proportion than is ever likely to be encountered in urine, as its reducing power seems to be relatively small, and is easily and entirely masked by a very small amount of kreatinin. Thus, a urine containing less than the normal minimum of kreatinin would require to have a great excess of uric acid present in order to give the reaction ; it is therefore probable that the reaction is seldom, if ever, dependant on uric acid. Glycuronic acid, on the other hand, does not seem to be present in appreciable quantities in urine, unless as the result of some drug which is being taken, so that dextrose is the substance most likely to give this phenomenon. In eight cases of urines giving this reaction more or less distinctly, I was enabled to demonstrate the fact, that dextrose was present to an extent beyond the usual average of 'normal' urine, and on the removal of the dextrose the reaction was not obtained ; in these cases, I corroborated the fact that sugar was the substance causing the phenomenon, by a combination and modification of Crismer's Safranine test with fermentation.<sup>1</sup> It would thus seem that, in the majority of urines giving this modified reaction, the phenomenon ought really to be ascribed to the presence of sugar and not to the action of other

reducing substances. On the other hand, the presence of a very small amount of kreatinin might result in the production of this phenomenon with the physiological trace of sugar present in normal urines ; other tests, however, demonstrate that, as a rule, it is due to a small excess of sugar. The question, however, of the significance of small amounts of sugar, while still unanswered, is yet, in many cases, of very material importance, both from a prophylactic and prognostic point of view. It is in such cases that Fehling's test is rendered obscure, and often difficult or impossible of interpretation, owing to the kreatinin present. If we take into account the number of ambiguous reactions yielded by Fehling's test in hospital practice, and generally ascribed to 'interfering' substances, but really usually due to small amounts of sugar, it is quite possible that the existence of small amounts of sugar beyond what may be considered the strict physiological limit is not so rare as is generally supposed. The constant presence of a minute percentage of sugar in normal urine has been demonstrated beyond question by many observers, and it is quite possible that occasional small amounts of sugar beyond what may be considered the normal amount may not be of much significance, more especially if observed early, and the conditions underlying its appearance modified or eliminated.

In the case of four healthy individuals whose urines were examined at short intervals—often two or three times daily—for some months, there was found undoubted evidence of small amounts of sugar in the case of two of these as indicated by Fehling's solution. This was substantiated by fermentation, and by the combination of safranine and fermentation mentioned above ; in both cases it was quite transitory, and no reasons could be given for its presence.

#### ON THE AMOUNT OF SUGAR WHICH MAY BE ADDED TO NORMAL URINE WITHOUT REACTING TO FEHLING'S SOLUTION

This seems to vary to a considerable extent, both in regard to different individuals, and in regard to the same individual under different conditions of food. In the case of a healthy adult living on

1. The exact manner in which this was accomplished will be fully described in a later number.

milk diet, it was found that the addition of '03 per cent. of sugar to the urine gave a greenish colourization at first, which, after some time, gave a fairly opalescent, dirty yellowish liquid ; about '06 per cent. gave a slight precipitate with Fehling's solution. When on ordinary mixed diet the addition of '03 per cent. of dextrose gave no change with Fehling's solution ; addition of '06 per cent. gave a green colour on boiling, resulting in a greenish, yellowish, opalescent mixture after standing. When living almost exclusively on a meat diet the addition of '08 per cent. of dextrose to the urine only resulted in a greenish tinge being obtained ; there was no opalescence on cooling. In the case of another urine, it was found that the addition of '03 per cent. of dextrose gave practically no change ; '06 per cent. gave a slight greenish-blue colour on heating, and, after cooling, an opalescent liquid. All the above only gave indications which would ordinarily be classified as due to 'interfering' substances.

#### ACTION OF KREATIN

It is doubtful whether kreatin occurs in normal urine, but in alkaline urine it is alleged that it may be sometimes present. In endeavouring to get rid of kreatinin I boiled the urine for some time with the alkaline part of Fehling's solution, in order to change it into kreatin. This urine, however, acted as before, and this led to the suspicion that probably kreatin acted in the same manner as kreatinin. This view was afterwards corroborated by the use of apparently pure kreatin,<sup>1</sup> this substance acting towards sugar in exactly the same way as kreatinin did.

#### ACTION OF KREATININ AND KREATIN IN MODIFYING THE COLOUR OF THE PRECIPITATE OBTAINED WITH FEHLING'S SOLUTION

In urines containing comparatively small amounts of sugar, but sufficient to give a distinct precipitate with Fehling's solution, it has often been noticed that the resulting precipitate tends to be yellow in colour (cuprous hydroxide) and not red as the reduced oxide. This appearance which, as observed by many investigators, is caused by

1. Samples were used in which no trace of kreatinin could be indicated by nitroprusside of sodium and alkali reaction.

kreatinin, may be obtained just as readily by the addition of a small trace of kreatin. This can be shown by adding kreatin to an aqueous solution containing sugar and treating with Fehling's solution in the ordinary way. Thus the experiment of Cipollina<sup>1</sup> by which a urine giving the yellow precipitate was, after long boiling with an alkali, found to give a red precipitate, probably meant that the kreatinin normally present had been destroyed by the alkali ; if it were only changed to kreatin this would in no way influence the colour of the precipitate, which would still be yellow.

#### SUMMARY

Normal urine requires a very much greater amount of dextrose to give a reaction with Fehling's test, than is necessary to produce a distinct reaction in aqueous solution. The chief factor concerned in this is kreatinin, and not ammonia as suggested by Pavy. Increased amounts of sugar may be masked, within certain limits, by a corresponding increase in kreatinin, such as may be caused by change of diet.

The amount of uric acid present in normal urine is more than sufficient to react with Fehling's reagent in the absence of kreatinin. Kreatinin has a very powerful influence in preventing uric acid from giving a reaction with Fehling's test ; it is probable that uric acid is never present in large enough quantity to give a precipitate of cuprous hydrate or oxide in a urine containing even the minimum normal amount of kreatinin.

Kreatinin in the amount in which it is generally present in urine would not be likely to cause confusion in virtue of its direct reducing power.

Many of the greenish liquids obtained on heating urine with Fehling's solution are due to the influence of sugar.

The opalescent greenish milky fluid obtained in certain urines some time after boiling with Fehling's solution, depends essentially on the kreatinin present modifying the reaction of some other reducing substance ; as a rule this reducing substance is really dextrose, present in the urine in an amount greater than the average for 'normal' urine.

Kreatin apparently acts in the same way as kreatinin.

1. *Deutsche Med. Wochenschr.*, 1901.

## STUDIES IN THE CHEMICAL DYNAMICS OF ANIMAL NUTRITION

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### PART I

#### INTRODUCTION. PROBLEMS RELATING TO THE FUNCTION OF ALBUMENOUS FOOD-STUFFS IN THE GENERAL ECONOMY OF NUTRITION

In spite of the fact that metabolism occupies a very important place in the literature of modern physiology, but little is known with certainty of the fate of the products of tryptic digestion of the albumens after leaving the alimentary tract. Various theories have been propounded to explain the rôle of the nitrogenous food-stuffs in the general economy of nutrition, of which that suggested originally by Liebig and modified by Pflüger has been, perhaps, the dominant. According to this theory the products of digestion of the albumens are incorporated into the protoplasm molecules of the living cells, and there in a readily oxidisable form, held available for the supply of the energy needs of the organism.

In quite recent years however, another, and almost diametrically opposed hypothesis, has been regarded with much favour, and has found an admirable exponent, amongst others, in the person of Speck.<sup>1</sup> According to this hypothesis, the nitrogen is rapidly eliminated from the products of digestion of the albumens, and excreted in the form of urea, whilst the carbohydrate part of the molecule forms, on oxidation, the main source of the energy supply of the organism. The tissues themselves undergo degradation only under exceptional circumstances, as, for example, when the food supply is insufficient for the energy needs, or when the conditions of metabolism are abnormal, as is the case during fever, or when the oxygen supply is insufficient, or after phosphorus poisoning. These abnormal conditions will be discussed again later. According to Speck, therefore, we must distinguish between the metabolism of energy and the metabolism of tissue, and as the main source of energy is derived from the oxidation of the non-nitrogenous part of the molecule, the albumens should play a comparatively subordinate rôle in the general economy of nutrition. Some support is given to this conception by the recent researches of Sivén<sup>2</sup> and of Chittenden,<sup>3</sup> both of whom find that equilibrium can be maintained on diets that contain very much less nitrogen than was supposed to be necessary by the earlier investigators.

An important new view of the matter has been advanced recently by Folin,<sup>4</sup> which does not differ very materially from that advocated by Speck. Folin has shown that the change from a nitrogen-rich to a nitrogen-poor diet is followed by marked changes in the composition of the urine. Not only does the urea output diminish in actual quantity ; its relative quantity in comparison with the other nitrogenous constituents of the urine also diminishes. On the other hand, the creatinine output remains always constant, whether the diet be nitrogen-rich or nitrogen-poor. The neutral sulphur of the urine maintains a similar constancy. From these facts Folin concludes that metabolism can be considered under two heads, viz. :—as *endogenous* metabolism, which is due to a constant tissue waste (and is analogous, therefore, to the tissue metabolism of Speck), which is responsible for the constituents of the urine (creatinine and neutral sulphur) which do not

vary with the diet, and *exogenous* metabolism, which is responsible for the greater part of the urea, and other varying constituents. According to Folin then, the only nitrogenous matter necessary for the maintenance of equilibrium is that required to supply the waste represented by the endogenous metabolism. Any superfluous nitrogen is eliminated as rapidly as possible in the form of urea.

We have, therefore, widely different views as to the function of the nitrogenous constituents of the food-stuffs ; on the one hand we have the view that the products of digestion are built up into the tissues, and that, relatively, large amounts of albumen are necessary to maintain the organism in full bodily vigour ; on the other hand we have the view that only small quantities are necessary—just the amount that is required for the repair of the tissue waste which is continually taking place, and that the main part of the bodily energy is derivable from the non-nitrogenous constituents of foods.

Which of these views, if either, is correct ? What is the true function of nitrogen in the economy of nutrition ? With the object of obtaining an answer to these questions, the following researches were undertaken.

#### METHODS OF RESEARCH

Our ignorance is to be largely ascribed to the paucity of our methods of research. Most of the older theories are based on experiments which had for their object the determination of the balance between *ingesta* and *egesta*, either by the analysis of inspired and expired air (Zuntz, Speck, etc.), or by the estimation of nitrogen in food and excreta (Pflüger, Voit, etc.), or by the estimation of caloric values (Rubner, etc.), or by a combination of all these methods of experiment (Atwater).

The systematic determination of the distribution of nitrogen amongst the various nitrogenous constituents of urine by the method of Folin already referred to marks a distinct advance in the study of metabolism.

An attempt has been made in the following researches to throw some light on the mechanism of nutrition by an entirely different method. It was assumed that if the products of albumen degradation

be built up into the bioplasm, and held there in a form in which they could be readily eliminated for the supply of the energy needs of the organism, as demanded by the hypothesis of Pflüger, it should be possible to detect variations in the *immediate* post-mortem changes in the tissues, according to whether such tissues are derived from animals during digestion, or during a period of fasting. It was assumed that tissue of an animal with nitrogenous matter stored ready for elimination for the supply of energy needs, should show a more rapid post-mortem change than tissues derived from an animal during a period of fasting.

With the exception of the small intestine, which will be discussed in detail below, it was found, in the researches carried out in conjunction with Miss J. E. Lane-Claypon,<sup>5</sup> that but small change took place, as far as the nitrogen at any rate was concerned, during the first four hours of incubation with water; but that, in the case of the liver, after this so-called 'latent period,' a rapid degradation of the albumen set in (lasting generally from 4-6 hours), the rate of which depended on the state of nutrition of the animal at the time of death. This autolysis was more rapid in the case of the fasting than of the fed animal—the reverse of what might be expected if the hypothesis of Pflüger be correct. From the results obtained it appeared that the autolytic enzyme functioned by acting when the energy needs of metabolism were not satisfied by the food-stuff ingested. The results also furthered the hope that this method of research might be available as a general method for the study of metabolism. It was tested<sup>6</sup> by being applied to a special case of abnormal metabolism, viz., when animals are fed with thyroid glands, and results parallel in every respect with those got by other methods were obtained.

What is the mechanism now by means of which this autolytic enzyme acts? Does it exist in the tissue as a zymogen, from which the enzyme is liberated by the means of a kinase-like body, as in the case of trypsinogen, or is it always present in the tissue, but is prevented from acting during periods of full nutrition by the presence of inhibitory bodies derived from metabolites, which gradually disappear after ingestion of food?

The latter of these hypotheses seemed the more likely, and in the second paper on the subject of autolysis, it was suggested that the tissue stability of the liver was the resultant of the mass action of three sets of bodies, viz., the tissue itself, the metabolites, or bodies derived from metabolites, and the autolytic enzyme.

The following immediate questions then arose :—(i) In what way do the metabolites act? Do the products of tryptic digestion themselves inhibit the action of the autolytic enzyme, or is it the products derived therefrom which exert this inhibitory action? (ii) If such metabolites, or bodies derived therefrom, exert an action in the manner suggested, are they continually circulating in the blood-stream, or are they taken up in the tissues and acting intracellularly?

To obtain an answer to question (i) the influence of glucose, and of the products of tryptic digestion of caseine on the rate of autolysis of the liver were determined. For an answer to question (ii) the relative inhibitory action of sera from fed and fasting animals on autolysis was investigated. As a result of these experiments it was found that glucose exerted no action on the rate of autolysis, whereas the products of tryptic digestion exerted a marked inhibitory effect.

In the experiments on the action of sera, it was found that in one series no difference could be detected between the influence exerted by the serum of a fasting animal and that of a fed animal; in a second series, however, the inhibitory action of the serum of a fed animal was slightly greater. In this second series, the serum contained a larger amount of uncoagulable nitrogenous bodies. All experiments were carried out with sera diluted with a triple volume of normal saline.

As the result of the above-mentioned preliminary experiments, the question as to whether the products of tryptic digestion circulate in the organism acquires additional importance. That they inhibit autolysis there is little doubt: on the other hand experiments carried out with sera do not conclusively prove that the serum of a fed animal contains these products in quantity sufficient to shew a greater inhibitory power than the serum of a fasting animal.

A series of researches was, therefore, undertaken with the object of investigating the distribution of nitrogen in the tissues in the different stages of nutrition, and of determining the ratio of the nitrogen of the coagulable albumen to the total nitrogen. The difference between the total nitrogen and the nitrogen of the coagulable albumen has, throughout these researches, been termed 'residual nitrogen.' (Ger. Reststickstoff). This expression is more convenient than the older term 'extractives nitrogen,' for, as will be seen in the sequel, the latter, in part at least, appear to form an essential part of the tissue.

The problem of the residual nitrogen is no new one, and has been attacked by many investigators who have obtained varying results; their work will be referred to when discussing the experimental part of these researches. The contradictory results obtained are due, without doubt, to the faulty technique employed; for this reason a comparative investigation has been made of the value of two or three different methods: as a result a new method has been employed, which obviates the fallacies of the other methods. These researches are described in Part II.

Part III deals with the application of the method to the examination of serum, liver, and small intestine. As a result it was found that the tissues of fed animals do not contain more residual nitrogen than those of fasting animals. Other interesting results were obtained, the discussion of which is deferred to Part VI.

As it is obvious from the results obtained in Part III, that the products of tryptic digestion do not themselves directly act *in vivo* in inhibiting autolysis, a further search was made for agencies likely to bring about this result. These researches, with all the others bearing on autolysis, are described in Part V. It was found, finally, that acids cause autolysis of the liver without any previous latent period, whilst alkalis have a marked inhibitory action. Now it is known that ammonia is formed from the products of tryptic digestion in the alimentary tract, and stored up in the liver and other tissues. The quantitative relations of the amounts to the quantity of acid necessary to produce the maximum rate of autolysis have been

investigated. As a result of these researches, certain conclusions have been drawn as to the general chemical mechanism of nutrition, and as to the rôle played by the nitrogenous food-stuffs (Part VI).

Another problem, also bearing directly on the general chemical dynamics of nutrition has been investigated, and certain preliminary results obtained. Hofmeister has stated his belief that coagulable albumens can be synthesized from peptones or albumoses in the mucous membrane of the stomach. Glaessner, working in Hofmeister's laboratory, obtained experimental results tending to confirm this hypothesis. As, for reasons discussed in Part II, there are certain objections to the experimental method employed, and as, furthermore by the analytical processes used in this work, the coagulable albumen can be directly estimated, the researches of Hofmeister and Glaessner have been repeated. The experiments are described in Part IV. They do not confirm the results of Hofmeister and Glaessner.

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#### PART II

#### ON THE METHOD FOR ESTIMATING RESIDUAL NITROGEN IN SERUM AND TISSUES

Attention has often been called to the difficulty of precipitating albuminous bodies without at the same time removing from solution substances which are otherwise easily soluble in water. This subject has been investigated recently by Haslam, who has demonstrated certain fallacies in the methods devised for the separation of albumoses by fractional precipitation with salts. S. N. Pinkus (private communication) has obtained similar results.

In the previous work on autolysis in the papers already quoted, the nitrogen of the non-coagulable bodies has been estimated in the

filtrate after precipitating the albumen with trichloracetic acid (added when the mixture is boiling). As the experiments have throughout been comparative, and carried out under as nearly as possible like conditions, the method has been satisfactory enough.

In the determination of the residual nitrogen in tissues, it was not always possible to carry out the experiments under perfectly identical conditions ; it was necessary, furthermore, to determine the absolute quantities of residual nitrogen in each case. Preliminary experiments were therefore undertaken to test the reliability of older methods, and to determine how much the results varied under different conditions. For this purpose a mixture of a solution of Witte's peptone and serum was made, and this mixture was diluted with varying quantities of water ; it was found that the amount of residual nitrogen obtained after precipitating with tannic acid (in the cold) or with trichloracetic acid (boiling), varied quite appreciably with the strength of the solution in which precipitation took place ; it was found that more residual nitrogen was obtained in the filtrate when the precipitants were added to a dilute than when added to a concentrated solution. Tannic acid precipitates of course a certain part of the peptone ; trichloracetic acid, on the other hand, precipitates only a minute quantity if added to a sufficiently dilute boiling solution. On the other hand, trichloracetic acid, when added to the solution of the peptone-serum mixture above a certain dilution, produces a precipitate in such a finely divided state that filtration through filter paper is almost impossible. As a general result it was found that the trichloracetic acid precipitation method gives fairly accurate results when the strength of the solution was maintained within certain definite limits of concentration.

It was important, therefore, to have a method available which would allow the coagulation to be carried out under perfectly constant conditions, and at the same time give the residual nitrogen in absolute numbers.

For such a method I am indebted to the suggestion of S. N. Pinkus. He has shown that if anhydrous sulphate of soda be added to solutions of albumens or to tissues, the water is abstracted from the same with a very slight increase of temperature, and without any appreciable

alteration of the albumen. In this way the albumen solution or tissue can be dried (at a temperature not exceeding 38°) and kept without alteration for a considerable time. The tissue dried in this way can be coagulated, and the nitrogen of the coagulum directly estimated. The difference between the number thus obtained and the number obtained by estimating the total nitrogen in the albumen solution or tissue represents the residual nitrogen.

The method has been carried out (in the case of serum) in the following way:—A measured quantity of serum is poured into a flat-bottomed, glazed, porcelain dish. An equal weight (roughly) of anhydrous sulphate of soda is then added. The mixture is then allowed to stand for some time. The anhydrous sulphate of soda is converted into the crystalline hydrated form, and the water is thus abstracted from the serum. The dried mass can be readily removed from the flat-bottomed dish by a knife (this is the reason for using a flat-bottomed dish instead of an evaporating basin) and powdered in a mortar. When finely powdered it is transferred to the Kjehldahl flask in which the incineration with sulphuric acid is carried out (a 700 c.c. round-bottomed Jena flask was always used for this purpose). Any small amount of the powder remaining in the porcelain dish or mortar can be removed by rubbing the vessels with small fresh quantities of anhydrous sulphate of soda, which is then added to the main quantity. The albumen is then coagulated by boiling the mixture in the Kjehldahl flask with absolute alcohol (100 c.c. alcohol for 15 c.c. serum, or 50 c.c. alcohol for 5 gr. tissue) for half-an-hour with reflux condensor. The alcohol is then decanted off on to a filter. Water is then added to the powder remaining in the flask (300-400 c.c.) and the mixture is then heated on a water bath. By this means the sulphate of soda and non-coagulable bodies are dissolved up and the coagulum remains suspended in the solution in the form of a fine powder. The water is decanted off through the same filter as that used for the alcohol, and the greater part of the coagulum remains behind in the Kjehldahl flask. This is again heated with water on a water bath, and the hot water is again decanted off through the filter. When the washings are free from sulphate of soda and have run through the

filter the filter paper containing small quantities of the coagulum is transferred to the Kjehldahl flask. Sulphuric acid is then added, the mixture incinerated and the nitrogen determined in the usual way.

In the experiments with serum described in Part III, 15 c.c. were always used for each experiment. The total nitrogen was estimated in this quantity by incinerating with 30 c.c. concentrated sulphuric acid; a filter paper was added to the mixture, so that the total coagulable albumen nitrogen was always determined under the same conditions. Another 15 c.c. were used for the determination of the coagulable nitrogen in the way just described.

For the examination of the tissues 5 grams of material were used for each determination. The finely divided tissue was weighed on a photographic balance (accurate to about 0.02 of a gram). It was then transferred to a flat-bottomed porcelain dish with a knife, and mixed up by means of the knife with a little more than an equal weight of the sulphate of soda. The glass pan of the balance was carefully cleansed by rubbing up with fresh quantities of sulphate of soda, and the mixture kept until wanted. The mixture was then powdered in a mortar, coagulated with alcohol, washed, and the nitrogen of the coagulum determined in the way already described for serum. The total nitrogen was determined in 5 grams of fresh tissue. Thirty c.c. concentrated sulphuric acid were employed for incineration in each case. A filter paper was added in the determination of the total nitrogen as in the case of the serum.

When working with solutions of albumens the same method can be employed. A little more sulphate of soda is added than the weight of water present.

The following series of experiments illustrate the foregoing remarks. In the last series the coagulable albumen is determined in a serum; the same serum is mixed with a concentrated solution of Witte's peptone, and the coagulable albumen determined in the mixture. These experiments were carried out very rapidly, and although the coagulum was washed only twice by decantation, it will be observed that only a minute quantity of the peptone nitrogen has been carried down. Far larger quantities of water-soluble bodies were present in this case than are ever met with in tissues.

## EXPERIMENTS

Determination of non-precipitable nitrogen in a mixture of sheep's serum and 10 per cent. solution of Witte's peptone after precipitating with tannic acid (Hedin's<sup>3</sup> solution). The nitrogen estimated in an aliquot portion of the filtrate.

## Series I

	Serum c.c.	Water	Strength of serum solution %	Peptone solution c.c.	Hedin's solution c.c.	N in filtrate c.c. $\frac{N}{10} H_2SO_4$ required
a	25	25	.50	5	25	20.1
b	25	75	.25	5	25	20.2
c	25	175	12½	5	25	22.8
d	25	375	6½	5	25	24.8
e	25	75	.25	5	25	21.1

In experiments a, b, c, d, the peptone solution was added to the diluted serum before adding the tannic acid. In (e) tannic acid was added to the serum before the peptone solution. These experiments shew that the amount of residual nitrogen obtained varies with the concentration of the solution in which the precipitation is carried out.

Series II. Same as I, with another serum, and another 10 per cent. peptone solution. The total N is represented by the following numbers :—(a) 24.0, (b) 26.0, (c) 27.9, (d) 28.6.

## Series III. With trichloracetic acid precipitation.

(a) 25 c.c. serum + 25 c.c. water + 5 c.c. peptone solution.

The mixture was then boiled. 25 c.c. of 10 per cent. trichloracetic acid were then added to the mixture whilst still hot. Filtrate was washed with hot water; it was then removed from paper and washed again with hot water. Nitrogen estimated in total washings.

Result :

$$N = 49.0 \text{ c.c. } \frac{N}{10} H_2SO_4$$

(b) 25 c.c. serum + 75 c.c. water + 5 c.c. peptone solution.  
Boiled. 25 c.c. trichloracetic acid solution added.  
Manipulation as in (a).

Result :

$$N = 51.8 \text{ c.c. } \frac{N}{10} H_2SO_4$$

Here again variation with dilution. Greater dilution impracticable owing to difficulty of filtration.

*Series IV.* Sodium sulphate method. Same serum and peptone solution as in Series III.

25 c.c. serum added to 5 c.c. peptone solution.

$$\text{Total N} = 490 \text{ c.c.} - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

$$\text{Coagulable N, (Expt. I)} = 435.7 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

$$(\text{Expt. II}) = 434.7 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

$$\text{Residual N} = (490.0 - 435.2) - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ} = 54.8 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

$$\text{N in peptone solution alone} = 47.4 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

Hence residual N in serum = 7.1 c.c.  $\frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}$ ; a number which subsequent experiments shows to be a trifle low.

*Series V.* Sodium sulphate method. Determination of N in coagulum.

$$15 \text{ c.c. of serum. Coagulable N} = 116.2 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

$$15 \text{ c.c. of serum} + 10 \text{ c.c. 10 per cent. Witte's peptone.}$$

$$\text{Coagulable N} = 118.5 - \frac{N}{10} \text{ H}^{\circ}\text{SO}^{\circ}.$$

Here, in spite of large amount of residual N from the peptone, very little is carried down with the coagulum.

The method gives, therefore, accurate results, and can be made independent of the concentration of a solution, as a dried powder is always used for coagulation.

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### PART III

#### THE DISTRIBUTION OF NITROGEN IN THE SERUM, LIVER, AND MUCOUS MEMBRANE OF THE SMALL INTESTINE DURING DIGESTION OF FOOD AND DURING A FAST

The method of research has been already described.

The liver was put through a fine mincing machine before weighing.

The intestine was cut out from below the pylorus to just above the ilico-coecal valve. It was opened up longitudinally and nailed down on a wooden board. The surface of the mucous membrane was carefully cleansed with cotton wool, and the mucous membrane was scraped off with the blunt edge of a knife. Scrapings were made alternately from the cardiac and the coecal end, and put into a tube and thoroughly mixed with a knife. The tube was stoppered until it was required for weighing out. The weighings were all made as soon as possible after killing the animals.

For the determination of total nitrogen the tissue was mixed with sulphuric acid in a Kjehldahl flask immediately after weighing. The portions of tissue required for estimation of the nitrogen of the coagulable albumen were mixed with sulphate of soda immediately after weighing. This mixture could be kept till wanted.

The animals used were always cats, except when otherwise stated. They were fully anaesthetized by A.C.E. mixture, and bled to death by opening the carotid artery when under the influence of the anaesthetic.

The blood was collected in a tall cylinder and allowed to clot. The serum was then poured off into a narrower cylinder, and the corpuscles, which were decanted off with the serum, allowed to settle. The required quantities of serum were pipetted directly, either into the Kjehldahl flask or into the flat-bottomed dish, according to whether the total N or the N of the coagulum was to be estimated.

Various morphological details were noted, such as weight, weight of liver, length of intestine, etc. In order to know the concentration of residual nitrogenous bodies, the total solids were estimated. In the case of serum this was done by heating about 2 c.c. in porcelain crucibles, first at about 60° and afterwards in a Victor Meyer bath jacketed with toluol at 107° C. The mucous membrane and liver were treated in the same way; instead of using a crucible, however, they were dried after spreading out as much as possible on a watch glass. In this case the ordinary paired watch glasses were used. Heating was continued to constant weight. The fed animals were killed about five hours after the last meal. The fasting animals were killed when the stomach was empty. The animals were killed in pairs, a fed and fasting animal

being killed on the same morning or the same afternoon. This was to obviate any difference that might be caused by differences of hydratemic state of the tissues due to differences of temperature. The general results are given in the accompanying table (Table I). The following abbreviations are employed :—

F = animal fasting.

D = animal killed during active digestion.

N = total nitrogen, expressed in c.c.  $\frac{N}{10}$  H<sup>2</sup>SO<sub>4</sub> necessary for neutralization of NH<sup>3</sup>

R = residual nitrogen      "      "      "      "      "

N - R = nitrogen of coagulable albumen.

The results obtained are summarized below.

TABLE I\*

No.	Nutrition	Weight Kilos	SERUM			LIVER			SMALL INTESTINE			
			N	N-R	Solids %	Weight	N	N-R	Solids %	Length	Mucous Membrane	N
1	F	1.95	127.0	115.0	8.7	...	...	...	...	...	...	...
2	F	2.85	126.0	116.0	8.3	...	...	...	...	...	...	...
3	D	2.60	131.6	122.0	9.0	...	...	...	...	...	...	...
4	D	3.10	125.5	112.2	8.8	...	...	...	...	...	...	...
5	F	3.20	130.0	118.0	8.4	...	...	...	...	...	...	...
6	F	3.15	124.0	116.0	8.3	...	...	...	...	...	...	...
7	D	3.50	149.0	135.0	9.9	...	...	...	...	...	...	...
8	D	3.45	108.0	99.0	7.4	...	...	...	...	...	...	...
9	F	2.85	135.6	124.0	9.2	45.9	144.0	128.2	31.6	...	...	...
10	F	3.30	136.5	119.4	9.0	54.1	145.5	127.7	33.3	...	...	...
11	D	2.90	121.7	109.0	8.4	63.6	130.0	118.2	30.3	...	...	...
12	D	2.25	125.5	117.2	8.8	61.3	122.5	107.8	29.1	...	...	...
13	F	3.25	130.4	117.7	9.3	54.8	126.7	107.5	31.0	...	...	...
14	F	3.60	131.3	127.0	9.6	58.4	131.0	114.5	30.2	...	...	...
15	D	4.35	125.6	114.0	8.8	98.6	97.5	79.8	37.7	...	...	...
16	D	4.40	143.6	135.2	9.8	109.8	103.5	94.9	34.3	...	...	...
17	F	2.60	131.7	113.3	9.1	109.2	99.0	82.0	47.6	4'6"	11.6	111.0
18	D	3.50	133.3	120.0	9.4	80.0	109.5	lost	33.4	4'0"	11.7	109.0
19	F	3.00	122.8	111.0	8.4	64.4	107.0	86.4	40.1	5'6"	11.2	117
20	D	2.90	128.2	110.0	8.7	90.3	117.5	101.0(?)	31.2	6'0"	13.6	109.0
21	F	3.30	...	...	...	65.7	110.0	96.3	34.1	4'4"	16.0	112.0
22	D	3.30	...	...	...	84.3	102.7	87.2	27.7	4'10"	15.1	lost
23	F	2.85	...	...	...	56.7	124.0	104.2	30.3	...	14.6	111.0
24	D	2.90	...	...	...	79.3	123.1	107.3	28.9	5'0"	16.6	110.0
												73.6
												25.3

\* The nitrogen determinations refer to 15 c.c. of serum, and 5 grams of liver or of mucous membrane of intestine.

*Serum.* With reference to the influence of nutrition on the composition of the serum there are numerous researches, of which the most recent are those of von Bergmann and Langstein.<sup>1</sup> The question as to whether the products of tryptic digestion can be detected in the serum has, in spite of the number of works on this subject, been by no means definitely settled. Another question, which has given rise to considerable controversy, refers to the presence of albumoses and peptones in blood (bodies giving the biuret reaction). These have been claimed to be found by Emden and Knoop,<sup>2</sup> by Langstein,<sup>3</sup> and by Nolf.<sup>4</sup> Their existence, on the other hand, has been denied by Abderhalden and Oppenheimer,<sup>5</sup> and by Neumeister.<sup>6</sup> Now in Part II of these researches (see p. 129) it has been shown that when albumens are coagulated in concentrated solutions, the coagulum carries down with it a not inappreciable quantity of water soluble substances. On the other hand, when coagulation is carried out in dilute solutions the precipitate is so finely divided that satisfactory filtration is almost impossible. For these reasons it is not surprising that contradictory results have been obtained.

In nearly every case in these researches, etc., the filtrates obtained after coagulation, both alcoholic and aqueous, have been submitted to the biuret reaction. In no single case was a positive result obtained.

*Neither the liver nor the serum was found to contain a trace of albumose or peptone.* With regard to the residual nitrogen of the sera the results are evident from the following table:—

TABLE II

No.	Fasting animals		R	No.	Animals killed during digestion		
	N	N - R			N	N - R	R
1	127.0	115.0	12.0	3	131.6	122.0	9.9
2	125.5	116.0	10.0	4	125.5	112.2	13.3
5	130.0	118.0	12.0	7	149.0	135.0	14.0
6	124.3	116.0	8.0	8	108.0	99.9	9.0
9	135.6	124.0	11.6	11	121.7	109.0	12.7
10	136.5	119.4	17.1	12	125.5	117.2	8.3
13	130.4	117.7	12.7	15	125.6	114.0	11.6
14	131.3	127.0	4.3	16	143.6	135.2	8.3
17	131.7	113.3	18.4	18	133.3	120.0	13.2
19	122.8	111.0	11.8	20	128.2	110.0	18.2
Total 1295.3		117.9		1292.0		118.5	
Ratio $\frac{R}{N} = \frac{9.10}{100}$				$\frac{R}{N} = \frac{9.16}{100}$			

We see from these numbers, that both the total nitrogen and the residual nitrogen in 150 c.c. of serum, taken from ten different animals, are almost absolutely identical in quantity. We are bound, therefore, to conclude that there is *absolutely no evidence that the products of tryptic digestion as such, are circulating in the blood stream.*

The residual nitrogen, except in three or four cases, varies very little from a mean. The variations bear, however, no relation to the state of nutrition.

We can certainly conclude that it is not the products of tryptic digestion in the serum which exert any influence on the rate of autolysis of the liver.

The variations in the total nitrogen are influenced chiefly by the hydraemic condition of the blood. When the percentage of total solids is high, the total nitrogen is high, and *vice versa*.

### *The Liver.*

TABLE III

No.	Fasting animals		R	Animals killed during digestion			
	N	N - R		No.	N	N - R	R
9	144.0	128.2	15.8	11	130.0	118.2	11.8
10	145.5	127.7	17.8	12	122.5	107.8	14.7
13	126.7	107.5	19.2	15	97.5	79.8	17.7
14	131.0	114.5	16.5	16	103.5	94.9	8.6
17	99.0	82.0	17.0	22	102.7	87.2	15.5
19	107.0	86.4	20.6	24	123.1	107.3	15.8
21	110.0	96.3	13.7	...	...	...	...
23	124.0	104.2	19.8	...	...	...	...
Total	987.2		140.4	Total	679.3		84.1
Average	123.4		17.5	Average	113.2		14.0
Ratio $\frac{R}{N}$	$= \frac{14.2}{100}$			Ratio $\frac{R}{N}$	$= \frac{12.3}{100}$		

From this table, it is evident, that so far from the ratio of residual nitrogen to total nitrogen being larger in the case of animals during digestion than in fasting animals, it is smaller. The total nitrogen is also generally larger in fasting animals. There are two or three exceptions, as, for example, in the case of animals 17 and 19. It will be noticed, however, that in these two cases, the percentage of total

solids is very high (47.6 and 40.1 respectively). Furthermore, animal No. 17 weighs only 2.6 kilos, whereas the liver weighs 109.2 grams. These figures seem to indicate a very fatty liver, and probably this accounts for the abnormality. The numbers seem to indicate that in the fasting animals autolysis is already taking place, and that the tissue of the liver is degrading in order to supply the energy needs of the organism.

Here, again, there is no evidence that the products of tryptic digestion directly inhibit autolysis *in vivo*.

*The Mucous Membrane of the Small Intestine.*

TABLE IV

No.	Fasting animals			Animals killed during digestion		
	N	N-R	R	No.	N	R
17	111.0	71.3	38.7	18	109.0	71.5
21	112.0	80.5	31.5	20	109.0	74.0
23	110.0	72.0	39.0	21	110.0	73.6
Total	334.0	210.2		Total	328.0	108.9
Ratio $\frac{R}{N}$	$= \frac{33.0}{100}$			Ratio $\frac{R}{N}$	$= \frac{33.2}{100}$	

It will be noticed in the above table that the ratio of residual to total nitrogen is a constant, and independent of the state of nutrition of the animal. The concordance of numbers obtained from different animals is so close, that in five out of the six cases the analyses would have been satisfactory if they had referred to a pure homogeneous product.

Another point of considerable interest is the fact, that the percentage of residual nitrogen is considerably higher than that found in any of the other tissues examined. As the mucous membrane of the small intestine plays an important part in the general nitrogenous metabolism, this point is of great significance, and as will be shown below, the ratio is higher in the carnivora, in which the mucous membrane of the intestine has been examined, than in the herbivora. It appears to be a constant for each species of animal.

Only a part of this residual nitrogen is eliminated on simple coagulation by boiling the fresh tissue ; if, however, the tissue be

incubated with water, a gradual elimination takes place in four hours ; the degradation changes then cease. This phenomenon has been already described in the first paper (*Journ. Physiol.* 32, p. 159) ; it is to be distinguished from ordinary autolysis.

The physical constants determined show that very little change takes place on coagulating the moist fresh tissue ; no more, in fact than takes place in the case of the liver, which does not contain a large quantity of residual nitrogen, and which shews very small change during the first four hours of incubation with water. All these facts justify the conclusion that the greater part, at least, of the residual nitrogen of the small intestine is not mere 'extractive' nitrogen, but represents a part of the protoplasm molecule which is held in loose combination, and which is intimately connected with the processes of nitrogenous metabolism, as is evidenced from the fact that it is greater in quantity in the case of carnivorous animals than in that of herbivora. This point will be referred to again in the summary, (part VI).

*Comparison of the ratio residual nitrogen : total nitrogen in different animals.*

Dog—No. 1. Fasting	...	41.2	38.8 (average).
", 2. Fed	...	36.5	
Pig	...	...	32.3
Rabbit—No. 1	...	...	29.7
", 2	...	28.2	28.8
Sheep	...	...	27.6
Ox	...	...	19.2

In the case of the ox, the sample for analysis was not taken from the whole intestine.

In the rabbit, only small quantities could be obtained for each analysis ; these were accurately weighed on a fine balance.

#### SOME PHYSICAL CONSTANTS OF TISSUE EXTRACTS

The following determinations of physical constants were made. The results throw but little light on the method of combination of the residual nitrogen with the main part of the bioplasm. It might be

expected from the generalisations made below that changes other than those affecting the nitrogen take place in the tissues ; the results obtained show no parallelism between the physical and the nitrogenous changes.

Five grams of tissue were allowed to stand for a short time with 50 c.c. of cold water, made just acid with one drop of acetic acid. A part of the extract was, in each case, heated rapidly to 100°, in a closed vessel (so that no evaporation took place), and maintained at this temperature in a boiling water bath for 15 minutes. The determinations were made with boiled and unboiled extracts.

*Liver. Electrolytic conductivity*

Unboiled extract	$R = 495.2$ , $c = 651.670$ , $k = 1317$
Boiled extract	$R = 443.5$ , $c = 651.670$ , $k = 1469$
Difference	...

$152$

*Small Intestine. Electrolytic conductivity*

Unboiled extract	$R = 320.9$ , $c = 651.670$ , $k = 2031$
Boiled extract	$R = 290.6$ , $c = 651.670$ , $k = 2242$
Difference	...

$211$

*Freezing point*

Unboiled extract	...	$\Delta = 0.08^\circ$
Boiled extract	...	$\Delta = 0.09^\circ$

Another experiment was made. Five Grams of tissue were boiled for half hour with 50 c.c. alcohol. The latter was decanted off, and the precipitate washed several times with hot water. The washings, both alcoholic and aqueous, were then evaporated to a small bulk in *vacuo*, and dissolved in water. This solution was made up to 32 c.c. The freezing point of this solution was then compared with the freezing point of an extract of 5 grs. tissue in 30 c.c. cold water.  
Result :

$\Delta$  For tissue extract ... =  $0.17^\circ$

$\Delta$  For extractives solution =  $0.17^\circ$

Attention has been called in an earlier paper to the fact that a considerable degradation of the small intestine takes place in the first four hours of incubation with water, which is followed by a period of small change. This phenomenon is discussed in detail in Part VI. The following experiment shows that the changes of electrical conductivity are not parallel to this change ; there is no sudden increase in the first four hours, followed by a period of no change.

The experiment was carried out with a suspension of 5 grs. tissue in 50 c.c. water ; temperature 40°. The conductivity of the boiled extract was also determined.

Time		Resistance
11.10	...	Incubation commenced.
11.18	...	267.6
11.33	...	257.1
11.48	...	252.0
12.15	...	242.5
12.30	...	236.0
1.27	...	199.0
2.5	..	170.5
2.40	...	145.1
3.13	...	126.0
4.20	...	94.4
5.0	...	82.5
Boiled extract.	Resistance	171.8

These results show a continuous decrease, which is due probably to changes other than nitrogenous degradation.

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## PART IV

THE QUESTION OF THE SYNTHESIS OF ALBUMENS IN THE MUCOUS  
MEMBRANE OF THE STOMACH

This question has already been referred to in the introduction.

The latest experiments on the subject, viz., those of Glaessner, conducted in Hofmeister's laboratory, were carried out in the following manner:—The mucous membrane of the stomach was stripped off and divided into two symmetrical halves. The one half was coagulated, and the amount of peptones and albumoses determined in the filtrate of the coagulum by the method of Pick. The other half was incubated in a moist chamber at 40° for three hours, and then coagulated, and the albumoses and peptones determined in the same way as in the unincubated half. Glaessner showed that there was a diminution of these bodies which reached a maximum when the animal was killed five hours after a meal.

The method employed in this research was similar; instead however, of using Pick's method, the coagulable albumen was determined directly by the procedure described in Part II.

## Results :

Dog A. Killed four hours after a heavy meat meal.

Non-incubated half.	Total N. 103.0	N - R 94.0	Ratio $\frac{N-R}{N} \times 100$	91.2
Incubated half.	," 105.0	," 92.2	,"	87.7

Dog B. Killed five hours after a meal.

Non-incubated half.	Total N. 108.5	N - R 96.8	Ratio $\frac{N-R}{N} \times 100$	91.2
Incubated half.	," 113.0	," 100.7	,"	89.1

The residual nitrogen was also determined in a fasting dog and found to be higher than in digesting dogs, *i.e.* as 23 : 97 (total N).

The changes taking place in a tissue during secretory activity are now forming the subject of another research. It is hoped to deal with these in a subsequent paper.

The above experiments give no evidence of a synthesis of albumens.

## PART V

FURTHER RESEARCHES ON AUTOLYSIS: THE RELATION OF  
AUTOLYSIS TO NUTRITIONSection A. *Autolysis of the Liver*

The method employed in the following researches is essentially that described in the first paper<sup>1</sup> (Lane-Claypon and Schryver, *loc. cit.*)

One or two minor modifications were made. After precipitating with trichloracetic acid, the mixture was allowed to cool. It was filtered when cold, after standing for several hours; the filtrate was washed with hot water and, in the event of the bulk not being too great, directly evaporated down with sulphuric acid in the Kjehldahl flask.

Except when otherwise stated, the numbers given under the heading 'autolysis' refer to the number of c.c.  $\frac{N}{16}$  H<sub>2</sub>SO<sub>4</sub> necessary for neutralisation of NH<sub>3</sub> obtained in the Kjehldahl analysis. Four grams of tissue were used for each experiment.

*Series I. Effect of Serum.*—In all the experiments with serum, a portion was incubated with saline as a control (same volume as liquid in actual experiment). After incubation, serum in same dilution (also incubated for the same time) was added to the control; this mixture was then rapidly heated to boiling, and trichloracetic acid in definite quantity added. The same amount of saline as was used for the control was added to the portion incubated with serum. The mixture was then boiled, and the same amount of trichloracetic acid added as was used in the control. By this means coagulation in the actual experiment and the control were carried out under absolutely identical conditions.

*Sheep's Serum on Cat's Liver.*—20 c.c. liquid to 4 grams liver.

Time of Autolysis	Serum %	Autolysis *	Control *	Difference
7 hours	100	18.8	31.2	12.4
7 "	50	15.5	26.0	10.5
9½ "	100	21.2	36.0	14.8
9½ "	25	17.7	28.5	10.8

A star indicates total N in filtrate from coagulum (includes residual N, N from products of tryptic digestion, etc.)

From these numbers it is evident that, whereas serum (even 100 per cent.) strongly inhibits the rate of autolysis, it does not entirely stop it.

*Series II.—Effect of sera of fed and fasting animals (cats) on autolysis of liver of a fasting animal.*

*Series (a).* Twenty c.c. of 25 per cent. solution of serum to 4 grams liver. Same liver used for experiment with fed as with fasting animal.

Time	Serum of fed animal			Serum of fasting animal		
	With serum*	Control*	Difference	With serum*	Control*	Difference
7 hours	16.3	20.0	3.7	16.5	16.2	0
9 hours	19.3	27.8	8.5	21.0	24.0	3
Total 12.2						Total 3.0

Here the serum of fed animal exerts an inhibitory action. Note, however, that the numbers under the heading 'control' are higher. This indicates that the serum of the fed animal contains a larger quantity of extractives.

*Series (b).*—Same as *Series (a)*. Liver and sera from different animals.

Time	Serum of fed animal			Serum of fasting animal		
	With serum*	Control*	Difference	With serum*	Control*	Difference
6½ hours	18.2	24.0	5.8	19.5	26.6	7.1
9½ hours	19.5	25.2	5.7	22.0	25.0	3.0
Total 11.5						Total 10.1

Here the same difference of the inhibitory action is not observed as in *Series II (a)*. From experiments on the composition of serum in Part III, one would not expect to find any very definite relationship between the inhibitory action of the serum and the state of the nutrition of the animal from which it has been obtained. The factors influencing autolysis will be demonstrated later.

*Series III.—Effect of the action of the products of tryptic digestion of caseine on autolysis.*

*Series III (a).*—Caseine in with twenty times its weight of water was digested in alkaline solution with trypsin. The mixture was then boiled and made just acid with acetic acid. Ammonia was then added until the reaction was plainly alkaline, and the whole was then concentrated by gently warming on a water bath.

The control was made by incubating with saline, and then adding the same quantity of products of tryptic digestion as was used in the actual experiment. The portion incubated with the products of digestion was diluted before coagulation with the same amount of saline as was used for the control. The filtrate in which N was estimated includes, of course, the products of digestion as well as those of autolysis.

5 c.c. trypsin solution = 36.0 c.c.  $\frac{N}{10}$  H<sub>2</sub>SO<sub>4</sub> (Kjehldahl)

20 c.c. saline + 5 c.c. to 4 grams of liver; control in 25 c.c. saline.

Time	With products of digestion	Control	Difference
7 hours	48.0	58.0	10.0
8 "	57.7	65.0	7.3
9 "	62.7	66.3	3.9

*Series III (b).*—To test the effect of the quantity of the products of digestion on autolysis rate. The control in this case represents the N of the filtrate obtained by boiling the tissue with the products of tryptic digestion, adding trichloracetic acid, etc., without previous autolysis.

T.D. = products of tryptic digestion. (5 c.c. = 36 c.c.  $\frac{N}{10}$  H<sub>2</sub>SO<sub>4</sub>)  
S = Saline

Solution used	Time	* N in filtrate	Autolysis
20 c.c. T.D. No saline.	0 hours (control)	147.5	...
	7 hours	153.2	5.7
	9 hours	157.5	10.0
10 c.c. T.D. + 10 c.c. S.	0 hours (control)	77.2	...
	7 hours	87.0	9.8
	9 hours	89.4	12.2
5 c.c. T.D. + 15 c.c. S.	0 hours (control)	38.7	...
	7 hours	53.3	14.6
	9 hours	57.2	18.5
5 c.c. T.D. + 35 c.c. S.	0 hours (control)	40.4	...
	7 hours	54.3	13.9
	9 hours	57.2	16.8

These figures indicate that the rate of autolysis is influenced by the quantity of the products of tryptic digestion, but little by the dilution.

*Series III (d).*—With another sample of the products of digestion of caseine the following results were obtained :—

Products of digestion alone  $N = 111.7$  c.c.  $\frac{N}{10} H_2SO_4$  (20 c.c.)

Time	With T.D. *	Control *	Difference
8 hours	128.5	138.0	9.5
24 "	140.0	135.2	4.8

Experiments were also made to determine whether previous treatment of the product of tryptic digestion with mucous membrane of the small intestine exerted any influence on the inhibitory power. No difference could, however, be detected.

*Series III (e).*—It will be shown below that acids accelerate autolysis, whereas alkalis inhibit it ; it was, therefore, of interest to determine whether the products of tryptic digestion inhibit autolysis in distinctly acid solutions, especially as it is somewhat difficult to be sure of the reaction to litmus of the products of digestion.

The sample of digestion products used had been prepared by digesting an alkaline solution (5 per cent.) of caseine for three months with Rhenania trypsin.

Amount of acid used for 4 grams liver = 20 c.c.  $\frac{N}{10}$  acetic acid

To this was added 10 c.c. T.D. solution = 95.5  $\frac{N}{10} H_2SO_4$

Time	With T.D. *	Control A *	Control B *
2 hours	106.0	116.3	115.5
4 "	115.5	129.8	131.0
6 "	123.3	144.5	144.5

Control A was made by boiling the mixture incubated with water before adding the T.D. solution ; then adding trichloracetic acid. In control B the T.D. was added first ; then the mixture boiled and trichloracetic acid added. These results clearly indicate the marked inhibitory action of the products of tryptic digestion.

*Series III (f).*—Attempts to reverse autolysis.

4 grams of liver in each case were incubated with 20 c.c.  $\frac{N}{10}$  lactic acid. After  $2\frac{1}{4}$  and  $3\frac{1}{4}$  hours respectively, the following mixtures were added :—

(a) 20 c.c. T.D. (of which N = about 190 c.c.  $\frac{N}{10}$  H<sub>2</sub>SO<sub>4</sub>)

(b) 20 c.c. T.D. + 20 c.c.  $\frac{N}{10}$  NaOH, so as to neutralize the lactic acid added.

(c) 20 c.c. T.D. + 25 c.c.  $\frac{N}{10}$  NaOH, so as to have a distinctly alkaline solution.

The effects of these solutions on the autolysed tissue, after acting for one and for two hours in the incubator, were investigated. In each case, instead of estimating the N in the filtrate, the N of the coagulable albumen was determined (by the method given in Part II), sulphate of soda being added in sufficient quantity to absorb all the water (*i.e.*, somewhat more than the weight of the whole mixture).

$$N \text{ of coagulum after autolysis with acid for } 2\frac{1}{2} \text{ hours} = 66 \text{ c.c. } \frac{N}{10} \text{ H}_2\text{SO}_4$$

Product of $2\frac{1}{2}$ hours autolysis + T.D.		after 1 hour	N of coagulum
" $2\frac{1}{2}$ " "	+ T.D. + 25 c.c. NaOH	" 1 "	64.2
" $2\frac{1}{2}$ " "	+ T.D.	" 2 hours	65.0
" $2\frac{1}{2}$ " "	+ T.D. + 20 c.c. NaOH	" 2 "	65.2
" $3\frac{1}{2}$ " "	+ T.D.	" 1 hour	63.0
" $3\frac{1}{2}$ " "	+ T.D. + 25 c.c. NaOH	" 1 "	63.0
" $3\frac{1}{2}$ " "	+ T.D.	" 2 hours	54.5
" $3\frac{1}{2}$ " "	+ T.D. + 25 c.c. NaOH	" 2 "	56.0

$$N \text{ of coagulum after autolysing with acid for } 4\frac{1}{2} \text{ hours} = 57.5 \frac{N}{10} \text{ H}_2\text{SO}_4$$

Although these results do not give any evidence of the reversal of the autolytic process, such an action is by no means improbable; there is no doubt of the powerful inhibitory action, which indicates that the reaction is probably reversible.

*Series IV.—Effects of products of peptic digestion.* In the following experiments, the autolysis was investigated in 20 c.c. saline to which were added 5 c.c. of a carefully neutralized 10 per cent. solution of Witte's peptone.

Time	With peptone *	Control *
0	67.3	67.3
2 hours	72.3	72.8
4 "	74.0	76.3
6 "	86.5	86.0
8 "	92.3	94.0
24 "	103.0	113.2

The peptone exerts, therefore, an inhibitory action only in the later stages.

*Series V.—The effects of acids and alkalis on autolysis.* When the main facts in the following series had been already discovered, a paper by Hugo Wiener appeared in the *Physiologische Centralblatt* (August, 1905) in which he suggested that the latent period of autolysis described in the first paper, was due to the fact that the tissues were still alkaline, and that the autolytic enzyme did not act until the tissues became acid (Cf. Hedin).

*Series V (a).*

ANIMAL I			ANIMAL II		
With acid		With acid			
Time	$H_2SO_4 \frac{N}{40}$ 20 c.c.	Control in Saline 20 c.c.	Time	$H_2SO_4 \frac{N}{40}$ 20 c.c.	Control in Saline 20 c.c.
0	8.7	8.7	0	11.7	11.7
4 hours	23.3	11.5	4 hours	25.4	15.6
6 "	40.0	22.2	6 "	33.5	21.1
8 "	44.7	29.2	8 "	41.0	31.7
10 "	50.7	37.0	10 "	45.8	38.5
24 "	61.8	55.8	24 "	52.5	49.5

*Series V (b).*

Effect of $NaOH \frac{N}{40}$ 20 c.c.		
Time	With alkali	Control
0	7.7	7.7
4 hours	9.1	10.5
6 "	9.7	12.0
8 "	13.8	18.3
10 "	15.4	25.2
24 "	26.3	34.2

*Series V (c).*

Effect of $Na_2CO_3 \frac{N}{40}$ 20 c.c.		
Time	With alkali	Control
0	10.6	10.6
4 hours	12.0	14.2
6 "	13.7	19.0
8 "	16.4	27.5
10 "	22.1	34.6
24 "	41.4	50.8

## Series V (a).

Effect of  $\text{NaHCO}_3 \frac{N}{40}$  20 c.c.

Time	With bicarbonate	Control
0	9.8	9.8
4 hours	11.2	10.9
6 "	11.8	14.5
8 "	19.5	22.5
10 "	32.0	31.0
24 "	43.0	50.7

As the result of these experiments it will be observed that acid causes a very marked acceleration of autolysis, whereas alkalis have a strong inhibitory action. Bicarbonate of soda acts as a weak alkali in this respect. The curves representing Series V (a) and V (c) are given in the accompanying diagram.

*Series V (c).—The effects of sulphuric acid with varying solutions or varying quantities on the rate of autolysis.*

In the following experiment 20 c.c. of  $\text{H}_2\text{SO}_4$  solution were used in every case:—

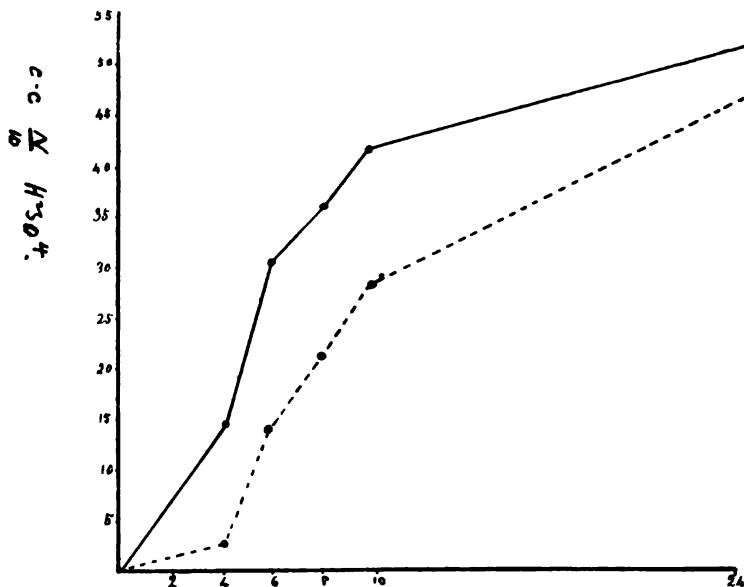


FIG. 1.

Time in hours.

Broken line: control in saline.

Effect of 20 c.c.  $\frac{N}{40}$   $\text{H}_2\text{SO}_4$  solution on autolysis of 4 grams of liver tissue.

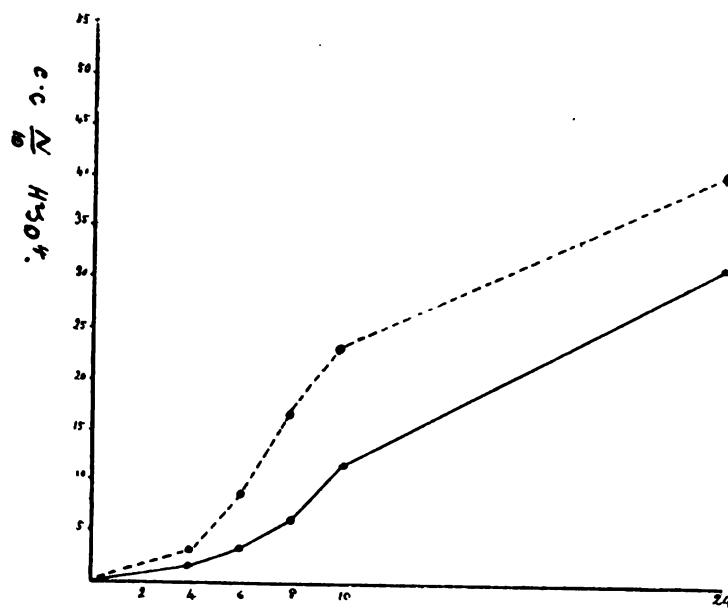


FIG. 2. Time in hours.

Broken line: control in saline.

Effect of 20 c.c.  $\frac{N}{40} Na_2CO_3$  on autolysis of 4 grains liver tissue.

Strength of solution	Amount of acid in 20 c.c.	Fasting animal		Fed animal	
		Autolysis 4 hours	Autolysis 6 hours	Autolysis 4 hours	Autolysis 6 hours
0	0.0	10.0	22.0	10.5	12.3
$\frac{N}{320}$	0.0030 gr.	9.7	22.3	11.8	12.8
$\frac{N}{160}$	0.0061 gr.	13.4	25.4	13.3	16.2
$\frac{N}{80}$	0.01225 gr.	19.5	33.3	17.5	26.2
$\frac{N}{40}$	0.0245 gr.	lost	38.5	22.7	32.5
$\frac{N}{20}$	0.049 gr.	26.4	40.7	24.8	29.5
$\frac{N}{10}$	0.098 gr.	22.8	31.0	21.5	27.2

We see from the above table a progressing rate of autolysis with an increasing strength of acid up to the concentration of  $\frac{N}{20}$ . Concentrations above this strength appear to be destructive to the enzyme.

1. By autolysis in this and the succeeding experiments, up to Series V (g), is meant the N in the filtrate after coagulation.

*Series V (f).—Is the rate of autolysis influenced principally by the strength of solution, or by the absolute quantity of acid present?*

To determine this point varying quantities of acids of varying strengths were employed.

Amount of acid	In c.c. solution	Strength	Autolysis 4 hours	Autolysis 6 hours
0	20	0	16.0	17.8
0	40	0	16.7	21.0
0.0245 gr.	20	$\frac{N}{40}$	26.3	...
0.0245 gr.	40	$\frac{N}{80}$	25.0	...
0.0245 gr.	60	$\frac{N}{120}$	25.0	35.3
0.049 gr.	20	$\frac{N}{20}$	24.8	...
0.049 gr.	40	$\frac{N}{40}$	24.9	37.9
0.049 gr.	60	$\frac{N}{60}$	24.5	38.3
0.98 gr.	20	$\frac{N}{10}$	20.0	35.0
0.98 gr.	40	$\frac{N}{20}$	22.0	39.0
0.98 gr.	60	$\frac{N}{30}$	25.3	42.2

These results indicate that provided sufficient acid is present (and it has been already shown in *Series V (e)* that 20 c.c. of  $\frac{N}{40}$  acid produces nearly the maximum result), the dilution in the case of a strong acid, like sulphuric acid, has but little effect. The action of the acid is akin, therefore, to the neutralization of a base, or the setting free of a weak acid from a salt, rather than to a phenomenon of hydrolysis. It is the amount of acid, therefore, that exerts the influence on the rate of autolysis rather than the concentration. There is, however, an inhibition with sulphuric acid when the concentration reaches  $\frac{N}{10}$ . This inhibition is due, as will be seen in the above table, entirely to the concentration and not to the quantity. It is probably, as already mentioned, due to hydrolysis of the enzyme.

*Series V (g).—The influence of organic acids on autolysis.*

In the experiments with acetic and lactic acids, the rate of autolysis with varying strengths and quantities has been compared with the rate in 20 c.c.  $\frac{N}{10}$  sulphuric acid.

The following experiments have been carried out with 20 c.c. liquid.

*Acetic acid.*

Amount of acid	Strength of acid	Autolysis in 4 hours	Autolysis in 6 hours
0	0	11.7	13.5
0.015 gr.	N 80	...	28.6 (?)
0.03 gr.	N 40	25.6	27.5
0.06 gr.	N 20	28.2	33.6
0.12 gr.	N 10	...	36.0
0.24 gr.	N 5	38.2	41.6
20 c.c. $\frac{N}{40} H_2SO_4$		27.8	28.2

*Lactic acid.*

Amount of acid	Strength of acid	Autolysis in 4 hours	Autolysis in 6 hours
0	0	15.7	23.3
0.0225 gr.	N 80	31.2	37.0
0.045 gr.	N 40	...	47.8
0.09 gr.	N 20	39.6	51.0
0.18 gr.	N 10	...	58.2
0.36 gr.	N 5	44.7	55.0
20 c.c. $\frac{N}{40} H_2SO_4$		27.3	41.0

We see from these figures that both acetic and lactic acid exerts a very powerful accelerating influence on the rate of autolysis, especially the latter acid. Lactic acid accelerates the autolysis more than does sulphuric acid in the corresponding strength of solution. This is important in view of the fact that lactic acid is known to be formed at times within the organism.

*Series V (h).—Influence of ammonia and trimethylamine on autolysis.*

In the following experiments the liver was incubated with the alkaline solutions in corked flasks. The autolysis was determined by estimating the nitrogen in the filtrate from coagulum in a portion before

incubation and a corresponding portion after incubation. Trichloroacetic acid was added before boiling to avoid loss of volatile alkali. The following numbers represent the amount of autolysis in  $7\frac{1}{2}$  hours :—

Solution used	Amount of Alkali	Autolysis
Without $\text{NH}_3$	0.	15.4
20 c.c. $\frac{N}{40} \text{ NH}_3$	.0085 gr. $\text{NH}_3$	8.3
20 c.c. $\frac{N}{20} \text{ NH}_3$	.017 " "	6.5
20 c.c. $\frac{N}{10} \text{ NH}_3$	.034 " "	5.4
20 c.c. $\frac{N}{5} \text{ NH}_3$	.068 " "	3.4
20 c.c. $\frac{17}{10} \times \frac{N}{10} \text{ N}(\text{CH}_3)_3$	.2008 gr. $\text{N}(\text{CH}_3)_3$	3.9

Here again we have a strong inhibition due to the alkali. This, as will be shown in the sequel, has an important bearing on the mechanism of nutrition.

*Influence of acids on the production of acid products of autolysis.*

Magnus-Levy has shown that when autolysis is carried out without antiseptics under conditions assuring asepsis, as far as can be determined by cultures, a considerable evolution of gas takes place (both  $\text{CO}_2$  and hydrogen), and certain quantities of fatty acids (amongst which is lactic acid) are produced. It has been noticed in the experiments recorded above that when acids (either sulphuric or an organic acid) are present, although nitrogenous degradation products are formed very rapidly, there is no evolution of gas, and no smell of fatty acids, even after 24 hours incubation. It would seem, therefore, that those conditions which favour the formation of nitrogenous bodies inhibit the formation of acid. It was also noticed that when the liver of a well-fed dog was autolysed in the presence of acid, the mixture at the end of 24 hours was still quite turbid with glycogen. The corresponding mixture in the case of a fasting dog was perfectly clear. This subject requires further research.

*Physiological action of the products of autolysis.*

Attempts were made to prepare a cytotoxic serum by the injection of cats' liver into the peritoneum of a rabbit. If the fresh liver be

injected in the form of an emulsion comparatively large quantities and repeated injections could be tolerated by the rabbits, without evincing any symptoms. On the other hand, when the liver had been incubated for only four hours (*i.e.*, till incipient autolysis), the animals invariably died, even after the interperitoneal injection of very dilute solutions. In one case an animal died within two or three hours of an injection.

These products when injected into the external jugular vein of an anaesthetized animal, of which the carotid artery was connected with a manometer, showed no change in the blood pressure.

The products of autolysis or the protoplasm of tissue in its unstable condition are evidently highly toxic. This subject is of considerable interest in connection with the toxæmic symptoms observed in atrophy of the liver. It is proposed to investigate this subject more completely.

#### *Section B. The autolysis of some tissues other than the liver.*

##### *Series VI.—Effect of acids on autolysis of kidney and muscular tissue.*

###### *A. Muscular tissue. (Quadriceps extensor of cat).*

Solution used	Water 20 c.c.	$H_2SO_4$ N 40 20 c.c.	Acetic acid N 5 20 c.c.	Lactic acid N 5 20 c.c.
4 hours	17.2	21.2	24.0	28.0
6 hours	16.8	21.2	24.0	27.0 (slight loss)

###### *B Kidney*

4 hours	16.7	43.7	55.0	53.4
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These results indicate that acid excites the autolytic enzyme in the case of the kidney. The influence of the acid in the case of the muscle is slight ; as there is no degradation between 4 and 6 hours the action appears to be a solvent one, rather than one exciting the enzyme.

##### *Series VII.—The influence of muscular activity on autolysis.*

In this experiment, the sciatic nerves of a cat were divided on each side as near the crural sciatic plexus as possible. The sciatic nerve on one side was tetanized for 25 minutes, and the animal was killed during tetanization (anaesthetic A.C.E.). The autolysis of the corresponding resting and acting muscles from the two legs was then determined.

Time	Resting muscle	Tetanized muscle
0	12.6	12.2
2 hours	13.2	12.7
4 "	15.8	14.1
6 "	16.3	...
8 "	17.4	18.0
10 "	22.4	23.4
24 "	34.2	32.8

The rate is practically identical for the two sides. The rate of autolysis does not appear, therefore, to be influenced by activity.

Experiments were also made on the rate of autolysis of fresh and frozen meat. No difference could be detected.

In the case of frogs' muscles, tetanization appeared to exert an inhibitory influence on autolysis. The difference was, however, but slight. No precautions were taken in these cases for asepsis, so that no definite conclusions could be drawn.

#### Series IX. *The comparative rate of autolysis in different tissues.*

The blood does not autolyse to any appreciable extent in 24 hours, either when incubated with ten times its volume of water, or in 0.5 per cent. ammonium oxalate solution. The thyroid gland also autolyses but to a very slight extent in 24 hours.

In the following experiments, the relative rate of autolysis of 4 grams from each organ in 24 hours was determined. Toluol was employed as an antiseptic.

Organs taken from two different dogs, given in table under columns I and II. In animal I, autolysis lasted 24 hours; in animal II, 20 hours.

Organ	Total N in 4 grams.		N <sup>I</sup>		N <sup>II</sup>		N <sup>II</sup> - N <sup>I</sup>		$\frac{N^I - N^I}{Total N} \times 100$	
			Nitrogen in filtrate before autolysis		Nitrogen in filtrate after autolysis					
	Animal I 24 hrs.	II 20 hrs.	I	II	I	II	I	II	I	II
Spleen	82.4	89.5	14.0	13.5	49.7	38.5	35.7	25.0	43.3	28.0
Liver	97.6	...	8.7	...	47.8	...	39.1	...	40.0	...
Kidney	76.5	...	14.5	...	32.8	...	18.3	...	23.9	...
Striated Muscle	95.3	99.5	9.8	12.0	25.0	26.2	15.2	14.2	15.9	14.3
Heart	80.0	87.4	10.2	11.2	27.6	33.7	17.4	12.5	21.7	14.3

It will be observed from this table that the maximum amount of autolysis occurs in the liver and spleen, the two organs which lose the greatest amount of weight during starvation. This affords a striking confirmation of the suggestion made in the first paper, viz., that the autolytic enzyme has the function of protecting the organism from starvation.

## PART VI

### A THEORY OF NITROGENOUS METABOLISM, DEDUCED FROM THE RESULTS OF THE FOREGOING RESEARCHES

Attention has already been called to the controversy as to whether the products of tryptic digestion of albumens can be detected in the blood ; it has been claimed, furthermore, that the methods hitherto employed for investigating this problem have not been entirely satisfactory. As it was imperative to arrive at a decisive settlement of this question, if possible, in order to gain a clear view as to the function of the albumens in nutrition, researches were undertaken with the object of obtaining a reliable method of chemical analysis, by means of which the nitrogen of the coagulable albumen could be accurately estimated ; such a method is described in Part II. The difference between the total nitrogen and the nitrogen of the coagulable albumen, designated throughout as 'residual' nitrogen, should, if the products of tryptic digestion circulate in the blood-stream, be greater in the case of animals killed during active digestion than it is in that of fasting animals. The method of research was also applied to a similar investigation of the liver and the mucous membrane of the small intestine. A large number of animals were used, and it was found then in no case was the average amount of residual nitrogen greater in the case of fed animal than in that of fasting animals.

The general conclusions drawn from the researches on residual nitrogen may be summarized under the following heads :—

I. The residual nitrogen, as deduced from the analyses of ten sera of fasting animals was found to be 9.10 per cent. of the total nitrogen ; corresponding analyses of the sera of ten fed animals gave the number 9.16. These figures are so close that they

may be regarded as practically identical ; the difference lies well within the limits of experimental error. The individual sera give numbers which do not differ very greatly from this mean.

Furthermore—*In no case was any substance yielding the biuret reaction detected in the filtrate from the coagulum*

II. In the liver, the percentage of residual nitrogen is larger (14.2) in the case of the fasting than of the fed animals (12.3). This result is probably due to the fact that autolysis was already taking place in the livers of the fasting animals at the time of death.

*In no case again were any bodies yielding the biuret reaction to be found in the filtrates from the coagulum.*

III. In the case of the mucous membrane of the small intestine, the following results were arrived at :

(a) The residual nitrogen varies within very narrow limits, if indeed, at all. The analysis made of six samples taken from six different animals gave in five cases numbers that agree so closely that had they referred to a single homogeneous body they could hardly have been more satisfactory. From this we may conclude that the percentage of residual nitrogen in the mucous membrane of the intestine is a constant for a particular species of animal.

(b) It is not influenced by the state of nutrition.

(c) It varies with different animals. It was found to be 38.8 in the case of the dog (two samples), 33.2 in the case of the cat (six samples), 32.3 in the case of a pig, 28.8 in a rabbit (two samples), 27.6 for a sheep, and 19.2 for an ox. It will be noticed therefore, that the percentage is higher in the case of carnivora than of herbivora.

(d) It is also higher in the case of the mucous membrane of the intestine than in any of the other tissues investigated.

(e) In addition, the mucous membrane shews certain other peculiarities. Attention has already been called to the fact that, unlike other tissues, when incubated with water it *immediately* commences to undergo disintegration.

This disintegration proceeds for three or four hours, after which in most cases it entirely ceases, at any rate for some time (Lane-Claypon and Schryver, *Journ. Physiol.* 31, pp. 173-4). In the researches just quoted, the residual nitrogen was determined in the filtrate from the coagulum, after precipitating with trichloracetic acid in boiling solution. The preliminary researches of Part II shew that when this method is used, nearly all the residual nitrogen passes into the filtrate, at any rate in fairly dilute solutions. Now the amount found by this method of analysis in the mucous membrane of intestine of five animals was equivalent to 17.8 c.c.  $\frac{N}{10} H_2SO_4$  for 5 grams of tissue if the residual nitrogen be determined before incubation. After incubation for 4 hours, however (that is to say after the first rapid disintegration had come to a standstill), this number rose to about 32 c.c.  $\frac{N}{10} H_2SO_4$ . This is very nearly the same amount as that found by coagulating the tissue, when dried by sodium sulphate according to the method described in Part II. This seems to indicate that part, at least, of the bodies represented by the residual nitrogen is in some form of chemical combination with the bioplasm.

What conclusions are to be drawn now from the above results?

In the first place, there is no evidence that the products of tryptic digestion as such can circulate in indefinite quantities in the blood-stream. How, then, are we to explain their fate after leaving the alimentary tract? What light do the above results throw on this subject?

Four sets of facts are of special significance in connection with these questions; they are:—

- (i) The percentage of residual nitrogen is very high in the mucous membrane of the intestine, a tissue most intimately connected with the nitrogenous metabolism.
- (ii) In the limited number of cases examined, it is higher in carnivora than in the herbivora.

- (iii) It is independent of the state of nutrition, and is the same in the fasting as in the fed animal.
- (iv) There is a certain amount of evidence that the bodies represented by the residual nitrogen are in a state of loose chemical combination with the bioplasm.

With these facts to guide us, the following explanation of the mechanism does not seem unreasonable :—

The bodies represented by the residual nitrogen may be regarded as in the same kind of chemical combination, such as exists between an enzyme and its substrate (or, perhaps, between a toxin and antitoxin); that in this state they undergo certain chemical changes like hydrolysis or oxidation, such as would take place through the action of an enzyme; the products of the change would be eliminated and carried in the blood-stream to other parts of the organism; after chemical change and elimination they would be replaced by other similar side chains, which would, in their turn, undergo the same kind of changes. The more rapid the blood-stream through the organ, the more rapid will the changes be.

According to this conception, then, the passage of the products of tryptic digestion through the mucous membrane is analogous to a continuous chemical process. The bioplasm acts as an enzyme or collection of enzymes, to specific points of which side chains are anchored; it keeps, furthermore, always saturated with side-chains, as is shown by the fact that the residual nitrogen is the same during digestion as during a fast.

The theory is analogous to that suggested by Verworn<sup>1</sup> to account for the utilisation of carbohydrates.

The question now arises: how does the bioplasm maintain its saturation with the side-chains in the absence of ingested food-stuffs?

To obtain an answer to this question, reference must be made to the researches on autolysis and on the products of metabolism carried from the digestive tract to the portal vein. The following sets of facts are known:—

- (i) The autolytic enzyme acts more rapidly in the liver of a fasting animal than in that of an animal during active digestion.

- (ii) It is inhibited by the action of ammonia and other alkalis, but accelerated by the presence of acids, especially lactic acid.
- (iii) This acceleration of acids is a function, in the case, at any rate, of sulphuric acid, depending on the *absolute* quantity of acid present, but not, except to a very limited extent on the concentration (Part V, Experiment Series V (f) p. 152).
- (iv) According to the researches of Nencki, Pawlow, and Zaleski<sup>2</sup>, ammonia is formed as a product of nitrogenous metabolism in the alimentary tract ; there is more ammonia in the portal vein than in any other part of the vascular system. The liver, furthermore, eliminates ammonia on treatment with weak alkalies, and more of this body is obtainable from the organ of a well fed animal than from that of a fasting animal. The numbers are lower in the case of herbivora than in the carnivora.

These facts offer a ready explanation as to the way in which the supply of products of albumen degradation can be maintained for the saturation of the bioplasm with nitrogenous side-chains in the mucous membrane of the intestine and other organs. Nencki, Pawlow, and Zaleski, show that the liver of a dog on treatment with weak alkalies at 40°C, yields on an average 26 mg. of ammonia per 100 gm. of tissue if the animal be killed shortly after a heavy meat meal, about 7.6 mg. if fed on bread and milk only, and about 7.3 mg. if killed during a fast.

The numbers are equivalent to 1.0 and 0.3 mg. NH<sub>3</sub> for 4 grams of tissue, quantities which require 0.0028 and 0.0008 gm. sulphuric acid for neutralization.

Now, on reference to Experiment Series V (e), Part V, (p. 150) it will be seen that in the case of cats, 20 c.c.  $\frac{N}{320}$  H<sub>2</sub>SO<sub>4</sub> containing 0.0030 gm. acid, a quantity only just sufficient to neutralize 1.0 mg. ammonia, exerts but little influence on the rate of autolysis, whereas the same volume  $\frac{N}{160}$  acid produces a marked acceleration. (There will, of course, be small quantities of sodium carbonate and other

alkalis in the tissues\*). Furthermore, it has been shown, that in the case of sulphuric acid, it is the absolute quantity rather than the dilution which increases the rate of autolysis.

From these results we arrive at the following conclusion as to the function of nitrogenous foodstuffs in nutrition :—

*In order to maintain nitrogenous equilibrium, nitrogenous food stuffs must be ingested in such quantities, and in such form that the ammonia produced therefrom in the digestive tract is sufficient to maintain the intracellular alkalinity of the liver and probably other tissues.*

In order to fully understand this mechanism it is of importance here to consider the products other than nitrogenous bodies which are formed during autolysis. Magnus-Levy<sup>3</sup> has shown that the liver on autolysis under the most stringent precautions for asepsis yields carbonic and other organic acids, such as lactic acid ; furthermore, the degradation products of fats and carbohydrates are of acidic nature—all would on hydrolysis or oxidation produce acids ; it matters not, however, for the purposes of the present argument whether the acid products are produced from the stored-up food-stuffs, or from the bioplasm itself.

We have, therefore, two classes of products producible, viz., non-nitrogenous acidic bodies and nitrogenous bodies ; the production of the latter class, it has been shown, is stimulated by the presence of the former. In well-nourished animals there is, however, always an excess of ammonia present, hence in this case the tissues will not become acid *in vivo*. The excess disappears gradually, however, if the animal is deprived of food. A certain stage will then be reached, when the production of acid exceeds the amount of ammonia available for neutralization ; the autolytic enzyme then comes into play, liberates amino-acids, etc., which in their turn pass the alimentary tract, and by means of the metabolic processes there taking place liberate ammonia, which again inhibits the production of nitrogenous degradation products. Degradation of tissue should proceed, therefore, at a definite uniform rate. From the preliminary experiments carried out it seems that the production of acids is inhibited by the presence of acids ; we have,

\* Ammonia is also responsible for the production of sodium carbonate ( $\text{NH}_4^+$ )<sub>2</sub> $\text{CO}_3$  + 2  $\text{NaCl} = \text{Na}_2\text{CO}_3 + 2 \text{NH}_4\text{Cl}$ .

therefore, a mechanism which tends to prevent excessive acidity or alkalinity of the liver ; in the presence of acid nitrogenous degradation products are formed, from which ammonia is produced in the digestive tract ; it is possible also that the presence of alkali stimulates the production of the non-nitrogenous acid bodies. Further research is needed on this point.

The production of nitrogenous degradation products proceeds also at such a rate that there is never a dearth of nitrogenous bodies in the blood stream, and the bioplasm of the tissues, especially the mucous membrane of the small intestine, can remain saturated with side chains, for we have seen that the liver of a fasting animal contains more residual nitrogen than does that of a well-fed animal.

From these researches, it follows, that while the animal derives most of its energy from the oxidation of carbohydrates, and rapidly eliminates nitrogen from albumens, the latter, for all that, play an important rôle in nutrition, for the degradation products are needed, not only to satisfy, as Folin has suggested, the needs of endogenous metabolism of the organism representing the wear and tear of the tissues, but also to supply sufficient ammonia to maintain a certain amount of general intracellular alkalinity. In the absence of this amount, nitrogenous equilibrium ceases to be maintained.

It remains, lastly, to consider the bearing of these conclusions in certain abnormal cases, resulting in what Speck has called tissue metabolism (Nahrungstoffwechsel as contrasted with Kraftstoffwechsel).

Four instances are cited by Speck, in which tissue degradation sets in, viz. : (i) lack of oxygen ; (ii) poisoning with phosphorus, arsenic, etc. ; (iii) withdrawal of water ; (iv) in fever.

The factors regulating the animal heat of the body are too little known to render it profitable to discuss the course of metabolism in fever ; whether high temperature is due to increased oxidation or not, is doubtful, as the results obtained by the investigation of the respiratory quotients are open to doubt. Little is known also of the course of metabolism in the absence of water.

The metabolism with lack of oxygen, and in phosphorus poisoning, have formed the subject of numerous researches, and several facts

have been discovered, which are in accord with the theory of metabolism just propounded.

The cases of deficiency of oxygen have been investigated by Fränkel and Geppert<sup>4</sup> and v. Terray<sup>5</sup>. The latter has shown that there is not only an increased nitrogenous output, but also an elimination of acids, such as lactic acid, in the urine. The cause of the increased nitrogenous output is here clear. Owing to lack of oxygen, the oxidation of carbohydrate does not proceed to the formation of the final product, carbonic acid, which is rapidly eliminated by the lungs; intermediary bodies, such as lactic acid, are formed, in the presence of which, the autolytic enzyme is brought into play, and so tissue degradation takes place. It is of interest to note, that increased nitrogenous output does not take place directly after a deficiency of oxygen; it generally follows in the succeeding days. This delay corresponds to the latent period of autolysis, and there is sufficient ammonia present to neutralize the lactic acid when first formed.

A similar explanation can be applied to the case of phosphorus poisoning, for Bauer<sup>6</sup> has shown that administration of phosphorus causes a diminished output of carbonic acid and a decreased oxygen consumption; it acts as a toxin to the oxydases.

We see from the above researches that the autolytic enzyme functions, by setting up tissue degradation when the food supply is insufficient for the energy needs of the organism; in this case, these needs are supplied by the utilization of degradation products of the tissue. *In this case autolysis is a strictly physiological process.*

There are, however, cases in which the process is pathological, as for example, in atrophy of the liver, in phosphorus poisoning, etc. Such conditions can result, as the above researches indicate, from a variety of causes, such, for example, as insufficient respiration, local stasis, etc. The difference between physiological and pathological autolysis is probably quantitative rather than qualitative; in the former case, it always proceeds at such a rate, that the tissue, by the mechanism already demonstrated, never becomes strongly acid; in the latter case, it is possible that the acid is produced at such a rate, that

the liver tissue becomes strongly acid, and degrades so rapidly that its functions are thrown out of gear, as in the case of an Eck fistula, and that instead of taking up ammonia after it is formed in digestion, and converting it into urea, it allows this body to circulate in the blood-stream and be eliminated in the urine. It is in these cases accompanied by lactic acid ; the concurrent production of these two bodies is, in this case, no protective mechanism ; it is a process which is pathological in the strictest sense of the word.

In such cases, the above researches would seem to indicate as a remedy the intravenous injection of carbonate of soda.

Another point of interest is the cause of toxæmia in such cases. Preliminary experiments have shown that the liver in an incipient stage of autolysis is intensely toxic. It remains to be determined whether this toxicity is due to the products of autolysis. Further researches will be undertaken for the elucidation of this point.

It remains also to apply the methods and principles already enunciated to be investigated of pathological cases ; much light might be thrown on the pathogenesis of various diseases of the liver by the investigation of the autolysis, under various conditions, of organs from the *post-mortem* room. Such a field of research must be left to pathologists.

There are also various other problems which await solution. What is the mechanism, *e.g.*, of restitution of tissue after autolysis ? Preliminary researches [Part V, Series III (f) ] show that the products of tryptic digestion of caseine exert a powerful inhibitory effect on autolysis ; it is possible, as already suggested, that the process may be made reversible.

Light might also be thrown on various problems of metabolism by the production of anti-sera to autolytic enzymes, and the investigation of the physiological properties of these sera.

Lastly, much remains to be ascertained as to the functions of the residual nitrogen ; the investigation of the chemical differences of acting and secreting glands should throw some light on this subject.

It is proposed to continue research in these directions.

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## ON THE INFLUENCE OF CALCIUM SALTS UPON THE HEAT-COAGULATION OF FIBRINOGEN AND OTHER PROTEIDS

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Ringer and Sainsbury<sup>1</sup> found that soluble calcium salts (chloride and nitrate) favour the heat-coagulation of serum proteids, causing a notable lowering of the coagulation temperature, etc., an action shared by salts of the allied elements, strontium and barium, and, to some extent, by magnesium sulphate. They found a certain amount of antagonism between calcium chloride on the one hand, and sodium and potassium chlorides on the other.

Ringer<sup>2</sup> also found that calcium salts have a remarkable influence in promoting the heat coagulation of alkali-albumin, while they have no such influence on egg-albumin.

As is well known, the heat-coagulation point of fibrinogen is remarkably definite and constant throughout the vertebrate series,<sup>3</sup> and it can be studied in its natural state in plasma, etc., with or without the possibly disturbing influence of acidification of the fluid. In view of the great importance of calcium in solution in the coagulation of blood and fibrinogen solutions, and the curious resemblances between the behaviour of fibrinogen in ferment-coagulation and in heat-coagulation—the splitting of the fibrinogen molecule in each case described by Hammarsten, etc.<sup>4</sup>—it seemed very possible that some relation might obtain between calcium salts and heat-coagulation. This has been found to be the case, though perhaps not in the direction that might have been expected.

1. *Journal of Physiol.*, Vol. XII, p. 170.

2. *Journal of Physiol.*, Vol. XII, p. 378.

3. See table in Haliburton's *Text-Book of Chemical Physiology*, p. 248.

4. *Arch. f. d. ges. Physiologie*, Vol. XIX, p. 563.

A large number of plasmas of different kinds were examined, without and with acidulation ; also hydrocele fluid, etc. Halliburton's heat-coagulation method was employed. The rate of heating was made to vary widely, from a rise of  $1^{\circ}\text{C}$ . per minute to three times that rate, and the results were essentially the same in all cases.

Some experiments were performed to try the effect of keeping the proteid at a temperature some degrees below coagulation point for prolonged periods, as there has been some difference of opinion in regard to the influence of the rate of heating upon the coagulation temperature of proteids.

Oxalate and citrate plasmas kept for twenty hours in a warm chamber at  $40^{\circ}\text{C}$ . were found to coagulate at the same point as before, and sodium sulphate plasma kept 24—48 hours at  $40^{\circ}\text{C}$ . showed no appreciable difference.

**NOTE.**—Horse's blood and ox blood show remarkable differences in regard to the rate of subsidence of the corpuscles in decalcified blood and in sodium sulphate blood respectively. In horse's blood the corpuscles sink rapidly in decalcified blood, and a large amount of clear plasma is soon obtained ; in the salted blood the change is much slower, and much less clear plasma is got. In ox-blood the converse holds good. Measurement of the depth of the layer of clear plasma in each case gave the following relative values :—

Horse's blood after standing 1 hour :	Oxalate blood,	65
" " "	Citrate " "	44
" " "	Sod. sulph. " "	0
" " 2 hours :	Oxalate blood,	69
" " "	Citrate " "	61
" " "	Sod. sulph. " "	10

Ox blood, after standing 1—6 hours : no plasma in decalcified or salted samples. After standing 24 hours, the depths of plasma in oxalate, citrate, and sod. sulph. blood were in the relation of 2, 4, and 10 ; after standing 72 hours the relations were as 7, 10, and 22.

Plasma from horse's and ox blood decalcified by addition of  $12-15$  per cent. sodium oxalate was found, when heated without acidulation, to give heat coagulation of fibrinogen at  $49.5^{\circ}-50.5^{\circ}\text{C}$ . with great constancy. After this coagulum had been filtered off there was no evidence of the presence of any fibrinogen remaining in the plasma ; no further coagulation at  $56^{\circ}-60^{\circ}$ , no ppt. on half-saturation with NaCl.

no attempt at coagulation on adding calcium salts, etc. No notable difference was seen in the behaviour of plasma faintly acidulated with dilute acetic acid before decalcification. Sodium sulphate plasma from the same blood heated alongside of the oxalate plasma showed the usual coagulation at  $56^{\circ}$  C. Potassium and ammonium oxalates gave results similar to sodium oxalate.

In the foregoing experiments the blood was decalcified by mixing with the oxalate when it was shed. In other cases salted plasma was first obtained, and then it was decalcified by adding oxalate. Essentially similar results were got by both methods.

20 c.c. of sodium sulphate plasma<sup>1</sup> (horse) were mixed with 3 c.c. of 1 per cent. sod. oxalate solution and filtered after some time; then heated alongside of a similar portion of the same plasma, pure or mixed with an equivalent amount of sod. chloride solution instead of sod. oxalate. In the former coagulation occurred at  $50.5^{\circ}$ , having become opalescent below  $50^{\circ}$ ; the latter coagulated at  $56^{\circ}$ , opalescent at  $55^{\circ}$ .

Sodium sulphate plasma faintly acidified with 2 per cent. acetic acid gave similar results, excepting that the oxalated portion sometimes showed a slightly higher coagulation temperature ( $51.5^{\circ}$  or  $52^{\circ}$ ) than usual; whether the acidification caused a little lime to remain in solution I cannot say. The non-oxalated sample coagulated at  $56^{\circ}$ .

Is the definite lowering of the heat-coagulation point due to the removal of the lime salts from solution in the plasma or to the presence of a certain excess of soluble oxalate? If the latter were the case, the presence of larger amounts of oxalate would probably increase the effect. This was tried by adding double and treble amounts of oxalate to the blood when shed and in other experiments by adding similar amounts to the ordinary oxalate plasma. All the samples were then heated in a water-bath along with one of the ordinary oxalate plasma (1.5 per cent.) and no appreciable difference was present in the time and manner of coagulation in the different tubes; the coagulation point was at  $49.5^{\circ}$ — $50.5^{\circ}$  C., while sodium sulphate plasma from the same blood coagulated as usual at  $56^{\circ}$ .

1. Obtained from blood mixed with half its volume of saturated sod. sulphate solution.

## RESTORATION OF SOLUBLE CALCIUM SALT

To test the effect of this, calcium chloride was carefully added to oxalate plasma till any excess of soluble oxalate present was used up and calcium was present in solution. But as such additions would readily lead to ferment coagulation the oxalate plasma was first mixed with sodium sulphate in sufficient amount to prevent spontaneous coagulation. It was found that restoration of lime in solution caused the coagulation point of fibrinogen to rise to the normal level. The addition of the sodium sulphate did not seem to have any disturbing effect, as shown by control experiments in which sodium sulphate was added without the calcium salt ; coagulation occurred as usual about  $50^{\circ}\text{C}$ .

## ADDITION OF MORE CALCIUM SALT TO PLASMA

The effect of adding more calcium salt to plasma was tried with sodium sulphate and sodium chloride plasmas, the latter obtained from blood mixed with an equal volume of 15 per cent or 20 per cent  $\text{NaCl}$  solution. There was some evidence of a slight raising of the coagulation above the usual point--at least with certain amounts of calcium salt. Thus when two drops<sup>1</sup> of 10 per cent  $\text{CaCl}_2$  solution were added to 10 c.c. sod. sulph. plasma, coagulation occurred at  $57.5^{\circ}\text{C}$ . ; after the addition of 10 drops, at  $59^{\circ}$ . Sodium chloride plasma (containing 10 per cent  $\text{NaCl}$ ) after the addition of 2 and of 10 drops showed somewhat similar results ; the coagulum does not separate so readily with the higher proportion of  $\text{CaCl}_2$ , and it is much less coarsely flocculent than usual. This plasma without addition of  $\text{CaCl}_2$ , coagulated about  $56^{\circ}$ .

When relatively large quantities of  $\text{CaCl}_2$ , were used (e.g., an equal volume or half-volume of 10 per cent.  $\text{CaCl}_2$  solution, there appeared to be a lowering of the coagulation point--to  $52^{\circ}$ - $53^{\circ}$ , etc.

Sodium fluoride plasma (from blood mixed with half its volume of 1 per cent. sod. fluoride solution) gave results similar to the preceding. Thus a sample from ox-blood gave coagulation at  $49^{\circ}\text{C}$ ., while

1. 14 drops=1 c.c.

sodium sulphate plasma from the same blood did not coagulate till the temperature rose to  $56^{\circ}$  (opalescent at  $54^{\circ}$ — $55^{\circ}$ ).

Sodium and potassium citrates gave similar results ; coagulation about  $50^{\circ}$ . The blood was mixed with half its volume or an equal volume of 1 per cent. citrate solution. Doubling or trebling the amount of citrate did not make any appreciable difference.

A combination of decalcifying agents was tried in some experiments ; citrate and fluoride were added to oxalate plasma. Coagulation occurred as before about  $50^{\circ}\text{C}$ .

In some experiments soap was used as a decalcifying agent, but the heat-coagulation was not satisfactorily determined ; there seemed to be a marked tendency for the fibrinogen to become changed into alkali-albumen.

#### PRECIPITATION OF FIBRINOGEN BY $\text{NaCl}$ IN DECALCIFIED PLASMAS

In oxalate plasma it was found that fibrinogen becomes precipitated by a percentage of  $\text{NaCl}$  much below the usual. Even 5 per cent.  $\text{NaCl}$  is sufficient to cause precipitation. In plasma mixed with an equal volume of 10 per cent.  $\text{NaCl}$  or correspondingly smaller amounts of stronger solutions a very considerable amount of stringy or membranous precipitate, with the ordinary microscopical characters of fibrinogen, separates out, though a good deal still remains in solution. Higher percentages of  $\text{NaCl}$  (7 per cent. and 10 per cent.) give more abundant precipitates. Citrate and fluoride plasmas were tested with similar results ; in all a very large number of plasmas from both horse's and ox blood were examined. Smaller percentages of  $\text{NaCl}$  fail to cause the precipitation ; less than about 5 per cent. is usually ineffective.

Such results present a marked contrast to what is seen in ordinary sodium chloride plasmas, apart from decalcification. Such plasmas containing 7 per cent. to 10 per cent.  $\text{NaCl}$  retain fibrinogen in solution for long periods, and are capable of ferment-coagulation after weeks or months.

Sodium chloride plasmas were decalcified by the addition of  $\frac{1}{8}$  vol. 1 per cent. sod. oxalate solution,  $\frac{1}{2}$ — $\frac{1}{4}$  vol. 1 per cent. sod. fluoride, a similar amount 1 per cent. sod. citrate, or sod. citrate crystals. With oxalate and fluoride a precipitate speedily appears, and separates out in an hour or two. With the citrate the separation is much slower, and there is often no visible change for hours. This fact was taken advantage of to compare the heat-coagulation of the plasma before and after the addition of citrate ; a lowering from  $56^{\circ}$  C. to  $51^{\circ}$ — $52^{\circ}$  was observed.

In the case of all the decalcifying agents a well-marked precipitate was found next day usually in the form of a very coherent shred or membrane lining the bottom of the test-tube. Stained microscopical preparations showed the usual characters of fibrinogen mixed with numerous particles of calcium oxalate, etc.

#### LEECH EXTRACT PLASMA

When leech extract or 'Hirudin' was used instead of oxalate, etc., to prevent coagulation, there was no lowering of the coagulation point of fibrinogen. Addition of 'Hirudin' to sodium sulphate or sodium chloride plasma—in amounts sufficient to prevent coagulation after dilution—showed no lowering when the plasma was heated either in the diluted or the undiluted state.

#### HYDROCELE FLUID

Decalcification caused heat-coagulation to occur at about  $50^{\circ}$ — $51^{\circ}$  C., as compared with  $56^{\circ}$  in the non-decalcified fluid. Some samples of strongly alkaline hydrocele fluid, poor in fibrinogen, were examined. When heated in the pure state these became opalescent at  $56^{\circ}$ — $60^{\circ}$ , but no visible separation of floccules took place. Similar samples treated with  $\frac{1}{10}$  vol. 1% sod. oxalate solution (without acidulation) coagulated at about  $51^{\circ}$ , numerous stringy flakes separating out. Similar results were obtained after faint acidulation. Addition to the non-oxalated fluid of amounts of sodium chloride equivalent to the

1. Supplied by Sachse & Co., Leipzig.

sodium oxalate used made no difference in coagulation point as compared with the pure fluid.

#### SERUM-PROTEIDS

Samples of serum were treated with sodium oxalate and allowed to stand for some hours, then heated along with samples of the same serum, pure or mixed with equivalent amounts of sodium chloride. No definite difference could be recognized in the behaviour of the various samples. They all showed opalescence at about the same, and coagulation at the same temperatures.

#### MUSCLE-PROTEIDS

Saline extracts of muscle were decalcified by oxalate, etc., and tested in the same way as plasma.

.75 per cent.  $\text{NaCl}$  extract of ox muscle ; 20 c.c. mixed with 4 c.c. sodium oxalate solution (1 per cent.) ; another portion mixed with an equivalent amount of  $\text{NaCl}$  solution. Abundant coagulation occurred in both tubes at  $47^\circ$ ; the coagulated flakes were much coarser, and perhaps separated more readily in the non-oxalated tube. Filtered and again heated. Coagulation in both at  $56^\circ$ , with the same apparent differences in detail.

5 per cent.  $\text{MgSO}_4$  extract of ox muscle treated as above. Coagulation in both between  $47^\circ$  and  $50^\circ$ ; floccules in the oxalated one were not so coarse and did not separate so readily in the oxalated sample. Second coagulation at  $55^\circ$ — $60^\circ$  in both.

5 per cent.  $\text{Na}_2\text{SO}_4$  extract of the same muscle gave similar results.

The relations of lime in solution towards the heat-coagulation of fibrinogen seem to be quite different from what has been so far observed in the case of other proteids and in notable contrast to the nature of the effects studied by Ringer and Sainsbury on serum proteids and by Ringer on alkali-albumin. In the latter instances, soluble calcium salt was found to have a remarkable influence in promoting the heat-coagulation of the proteids, lowering the coagulation

point, etc. With fibrinogen on the other hand the influence of lime is in the opposite direction ; the removal (or virtual removal) of soluble lime favours coagulation, causing a very decided lowering of the heat-coagulation point.

The foregoing inquiry was carried out on Professor MacWilliam's suggestion.

#### SUMMARY

1. Decalcification of blood, plasma, hydrocele fluid, etc., by oxalate, citrate, or fluoride, causes a lowering of the heat-coagulation point of fibrinogen from  $56^{\circ}$  to about  $50^{\circ}$ . Doubling or trebling the amount of decalcifying agent added makes no appreciable difference.
2. Restoration of the lime is followed by a rise of the coagulation point to about the normal level.
3. Addition of excess of soluble lime salt elevates the coagulation point somewhat.
4. Decalcification changes the fibrinogen in another way ; it is precipitated by an unusually low percentage of  $\text{NaCl}$ —beginning with 5 per cent.
5. No similar lowering of coagulation point was seen in solutions of muscle-proteids, egg-albumin, and serum-proteids. The effect (of rendering the lime insoluble) on fibrinogen is, as far as is known, a special one—in contrast with what Ringer and Sainsbury found in the case of serum-proteids and alkali-albumin where the addition of soluble lime salts favours heat-coagulation, lowering the coagulation point, etc.,
6. In the case of some other proteids (muscle-proteids, egg-albumin) no special effect upon the temperature of coagulation seems to be produced by the presence or absence in solution (as tested by decalcifying agents such as oxalates, etc.) of small amounts of lime salt that are normally present.
7. In regard to fibrinogen the relation of calcium salts to heat-coagulation is very different from their relation to ferment-coagulation.

ON SOME ASPECTS OF ADSORPTION PHENOMENA,  
WITH ESPECIAL REFERENCE TO THE ACTION OF  
ELECTROLYTES AND TO THE ASH-CONSTITUENTS  
OF PROTEINS

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I. THE GENERAL LAW OF ADSORPTION AND THE NATURE OF THE UNION BETWEEN PROTEIN AND ASH

The great difficulty of obtaining preparations of protein bodies free from ash-constituents is well-known to all observers who have had occasion to make the attempt to do so. It is, indeed, a matter of doubt whether a true protein absolutely free from inorganic electrolytes has hitherto been in the hands of investigators.<sup>1</sup>

It is easy by dialysis, or other means, to remove a portion of these constituents, but beyond a certain limit it appears to be a matter of extremely prolonged labour to get rid of the remaining small amount.

<sup>1.</sup> See *Die Grösse des Eiweissmoleküls*, by Fr. N. Schulz, 1903, p. 9.

One is therefore justified in some degree in distinguishing with Schulz<sup>1</sup> between the 'essential' and 'accidental' ash-constituents of proteins. The discussion as to the state of combination of these constituents in the protein molecule concerns in fact only the former, that part namely which it appears difficult or impossible to remove without destroying the character of the protein itself.

Now of recent years a considerable amount of research has been undertaken with regard to the nature of the phenomena known by the name of 'absorption' or 'adsorption'.<sup>2</sup> Too much space would be taken up in giving a complete list of this work, and I must content myself with mentioning the names of Van Bemmelen, Biltz, Schmidt, Walker and Appleyard, and Craw.

The main result of these investigations, so far as concerns us for the present purpose, is the discovery of what we may call the 'Law of Adsorption.' Suppose we have a series of solutions of such a dye as Congo Red, for example, in progressively diminishing concentration, and in each of these we place the same amount of filter-paper, we find that a part of the dye is taken up by the paper *and in relatively larger proportion the more dilute the solution.* To take some examples from the paper by G. C. Schmidt.<sup>3</sup>

**Picric acid and cellulose :**

Concentration of picric acid		Amount adsorbed
0.889	...	0.111
0.340	...	0.095

**Eosin and silk :**

Concentration of eosin		Amount adsorbed
0.666	...	0.84
0.376	...	0.75

**Iodine and carbon :**

Concentration of iodine		Amount adsorbed
0.161	...	3.275
0.087	...	2.958

1. *Loc. cit.*, p. 11.

2. Notwithstanding the suggestion of Zsigmondy (*Zur Erkenntnis der Kolloide*, Jena, 1905, p. 61, footnote) to make use of the name 'absorption' in honor of Van Bemmelen, the chief worker on the question who uses this form, I think that, in English at all events, 'adsorption' is less likely to lead to confusion, and 'absorption' can be still used for phenomena like the solution of gases in water.

3. *Zeitsch. f. Physik. Chemie.*, XV, p. 60. 1894.

These numbers show that there is some kind of 'affinity' between the bodies adsorbed and those which take them up. Put in other words the law states that there is not a proportionality between the concentration of the solution or partial pressure of the solute and the amount adsorbed. Ostwald<sup>1</sup> indeed points out that there is no hard and fast distinction to be drawn between chemical affinity and adsorption which latter he sometimes speaks of as 'mechanical affinity.' He calls attention to the complete series of transitions between the two phenomena, and, in referring to Van Bemmelen's researches, he remarks that they frequently leave it a matter of uncertainty as to whether the facts brought forward should be regarded as of a chemical or physical nature. In any case all the evidence shows that electrolytes when adsorbed are non-ionized and no longer take part in the electrical conductivity of the solution. This circumstance renders the determination of conductivities a rapid and convenient method of investigating certain aspects of the problem as will be seen later.

In the course of a series of experiments on the changes of conductivity in protein solutions brought about by the action of enzymes I have had occasion to take into consideration the possibility of the splitting-off of inorganic electrolytes under these conditions. The results of some preliminary experiments suggested to me that the most satisfactory explanation of the nature of the 'combination' between protein and ash was that the constituents of the latter are present in adsorbed form rather than in true chemical union.

Before passing on to the description of the experiments made to throw light on this problem, I may, in order to make clear the point of view, give the results of a preliminary experiment on an undoubted case of adsorption, viz., congo-red and filter-paper.

*Experiment:* Seven flasks were taken, each containing 50 c.c. of a solution of congo-red in 10 per cent. alcohol. These solutions were of a regularly diminishing concentration from 0.014 to 0.002 per cent. Congo-red forming a colloidal solution is very readily precipitated from watery solution by traces of electrolytes, so that an alcoholic

1. *Lehrbuch d. Allgem. Chemie.*, 2te. Aufl. Bd. I, p. 1084, et seqq.

solution is more easily worked with. For the same reasons, the filter-paper used was Schleicher and Schüll's analytical paper, and of this a disc 12.5 cm. diameter was added to each of the above flasks. After 24 hours standing the depth of colour in each solution was determined by Gallenkamp-Heele's colorimeter, the results being as follows:—

Concentration of solution	Proportion of dye in solution	Proportion of dye in paper
0.014	40 %	60 %
0.012	20 %	80 %
0.010	9.3 %	90.7 %
0.008	4 %	96 %
0.006	1.3 %	98.7 %
0.004	trace	practically all
0.002	"	"

The figures show at once that the amount of dye taken out of solution by the paper is not directly proportional to the concentration of the solution; nor, on the other hand, is it independent of this concentration as would be the case if a true chemical compound were formed. In fact, from a 0.014 per cent. solution  $\frac{40}{100} \times \frac{0.014}{2} = 0.0028$  gram is taken up by a certain amount of paper, whereas from a 0.008 per cent. solution only  $\frac{4}{100} \times \frac{0.014}{2} = 0.00028$  gram, or one-tenth of the former, is taken up by the same amount of paper, although there was still a quantity of free congo-red in the solution. The peculiarity of these phenomena, which I have spoken of above as 'the law of adsorption,' is well shown by the series of numbers in the third column.

Now, if we put these data into the form of a curve as is done in Fig. 1, where the ordinates represent the percentage of dye left in solution and the abscissae the original concentration of the solution, we see that the curve forms part of a hyperbola, and only approaches the axis (*i.e.*, zero concentration) asymptotically.<sup>1</sup> In other words, however diluted the original solution may be, there will practically be always a certain amount of the dye left unadsorbed by the paper. It follows

1. This hyperbolic form of the curve is pointed out by Ostwald, *Allgemein. Chem.*, Bd. I, p. 1096.

Walker and Appleyard (*Jour. Chem. Soc.*, 69, 1896), p. 1334, also find a logarithmic formula for the adsorption of picric acid by silk.

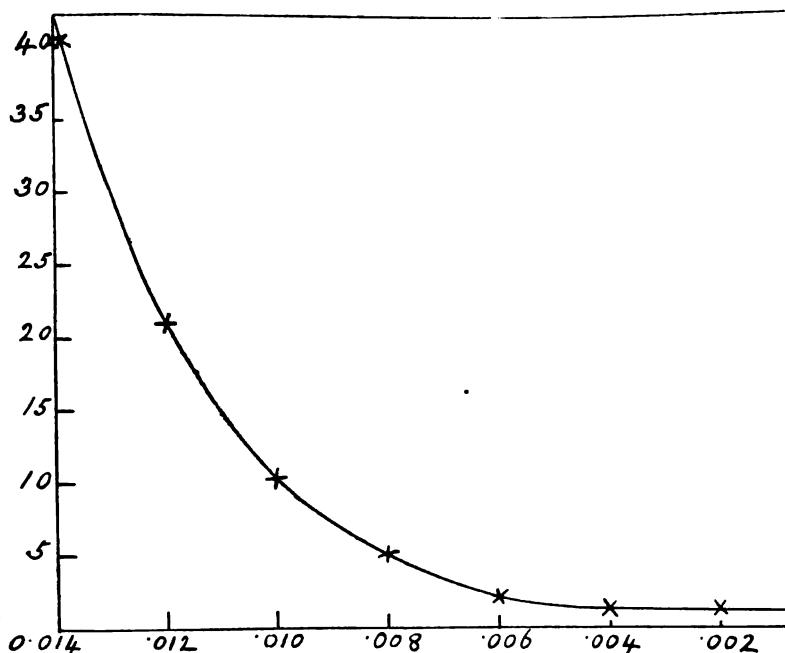


FIG. 1

also that if we attempt to wash out the dye by means of 10 per cent. alcohol it will require, theoretically, an infinite number of changes of the solvent to effect complete removal of the dye. I found, in fact, that if the piece of paper which was stained deep-red in the 0.002 per cent. solution was allowed to soak in 10 per cent. alcohol, a trace of dye only was removed each time a fresh supply of alcohol was added ; the alcohol became very faintly pink, the colour only to be detected in a deep layer, while the paper did not perceptibly lose in depth of colour after several extractions.

## II. WASHING-OUT OF ELECTROLYTES

It will be seen, on a little consideration, that the great difficulty of removing the last small part of the ash-constituents of proteins may be readily explained on the hypothesis that the case is one of adsorption. The hyperbolic form of the characteristic curve shows

that, as the asymptote is approached, a less and less percentage of these constituents will be taken away by repeated changes of the extracting solvent.

In order to test this hypothesis, I have taken commercial gelatin and extracted it with repeated changes of distilled water at room temperature. The comparative amounts of electrolyte removed by each change were estimated by taking the electrical conductivity. Gelatin at room temperature not being dissolved by water this latter can be poured off and replaced by fresh with ease. Of course, the water imbibed by the gelatin in swelling cannot be changed ; this does not affect the result to any serious degree, merely making the process of extraction a more prolonged one. The experiment was performed as follows :—10 grams of Coignet's gold-label gelatin were allowed to soak in distilled water until swollen and sufficient water added to enable 100 c.c. to be poured off. About 180 c.c. were needed in all. A little toluol was added, and the mixture left to stand for twenty-four hours. The water was then poured off and its conductivity determined. A fresh 100 c.c. of water were then added, and the process repeated every twenty-four hours. After the first three or four extractions the conductivity of the extract was so low that I found, with the apparatus used, that more accurate readings were obtained by concentrating the fluid in a platinum capsule over the water-bath to a definite volume (12 c.c.) before measuring its conductivity. Even when their concentration is thus increased, the electrolytes may be looked upon as practically completely dissociated, so that no appreciable error can be put down to this circumstance. It might be objected that impurities in the distilled water would also be concentrated, but I found on testing this possibility that the conductivity of the distilled water used, which had originally a conductivity of 5 gemmhos (= reciprocal megohms) did not increase by concentrating 90 c.c. down to 12 c.c. Presumably, this is to be accounted for by the fact that the slight conductivity was almost entirely caused by carbon dioxide, which would be driven off on heating. The numbers obtained were as follows :—

No. of extract	Conductivity in gemmahos	No. of extract	Conductivity in gemmahos
1	1094	...	7
2	475·5	...	8
3	232·3	...	9
4	120·4	...	10
5	62·8	...	11
6	29·41		
			23·25
			18·25
			15·94
			15·45
			15·40

We see at once that after a certain time each further change of water contracts only an infinitesimal amount of electrolytes. Since the conductivity of the distilled water was 5 gemmhos, this must be subtracted from the values given in the table, leaving only 10 gemmhos for the electrolytes washed out from the gelatin. These electrolytes consist in all probability chiefly of calcium sulphate, and a calcium sulphate solution of 0·25 per cent. has a conductivity of 2039 gemmhos, so that, neglecting differences of dissociation, a solution with a conductivity of 10 gemmhos will contain only  $0\cdot25 \times \frac{10}{2039} = 0\cdot0013$  per cent. It may be said that at this stage the ash was nearly all washed out. In order to test this possibility, I determined the ash of the gelatin. The incineration was performed in a platinum capsule, care being taken not to let the temperature get above dull redness, and not to prolong the heating after all charred portions had lost their blackness. The original gelatin contained 0·55 per cent. of ash, and after extracting with water eleven times it still contained 0·036 per cent. Therefore, when the rate of extraction had already become almost negligible, there was still left in the gelatin a considerable amount of ash.

On the hypothesis that the electrolytes are merely mixed with the gelatin and washed out by diffusion, the rate of diminution of conductivity of the successive extracts would have been much greater. This can be seen by calculating on the basis of the amount washed out by the first and second extractions, what would have been, e.g., the conductivity at the seventh extraction. The proportion removed at each change would, obviously, have been the same. If the above calculation be made it will be found that the conductivity of extract No. 7 would have been 7·4 gemmhos instead of 23·25 gemmhos as found,

Fig. 2 gives the curve of this experiment showing the same hyperbolic form as the adsorption curve of congo-red, Fig. 1.

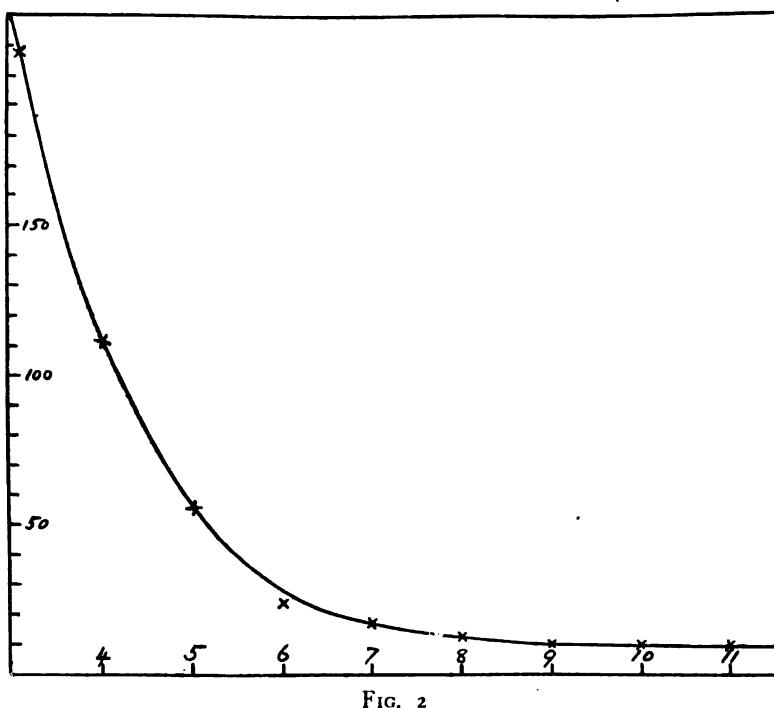


FIG. 2

Since the last values are the most interesting, and if the whole had been plotted out, the final course of the curve would have been on a much smaller scale than in the figure, the first two values are omitted, the curve commencing at the third. The ordinates represent specific conductivities of the extract in gemmhos, the abscissae are at equal intervals. The value No. 6 is obviously an experimental error. In drawing the curve the conductivity of the distilled water used is taken as the axis of abscissae. The position of the other asymptote is not shown since only part of the hyperbola has been drawn.

Before leaving this experiment I may mention that an attempt was made to re-dissolve the ash and determine its conductivity. A quantity of the gelatin after the last extraction was dried in a toluol oven and weighed 0.75 grams. This was incinerated and the ash redissolved in the quantity of water corresponding to that in which the

gelatin had soaked, viz., 13.5 c.c. It did not, however, entirely go into solution ; yet notwithstanding this, the conductivity was 29 gemmhos as compared with the 10 gemmhos of the water in which the gelatin had been soaked. The signification of this fact is that it confirms the deduction drawn from the previous experiment that electrolytes are held by the gelatin in such a manner that they can only be separated by an enormous number of changes of water.

If gelatin which has been washed free from the greater part of its electrolytes be placed in a dilute potassium chloride solution, it takes up a certain quantity of this electrolyte, which can be washed out by repeated changes of water, and the curve of the conductivities of the series of extracts is of exactly the same form as that of Fig. 2.

The fact mentioned in the last paragraph makes it of interest to see whether direct evidence can be obtained of the adsorption of electrolytes by gelatin. This can be done in the following way :—

*Experiment* : 30 c.c. of  $\frac{N}{10}$  KCl were found to possess a conductivity of 16,600 gemmhos at 39.7° C. At room temperature 2.3 grams of 'washed' gelatin were placed therein and allowed to remain for 24 hours. The conductivity of the solution was now found to be diminished to 14,590 gemmhos at 39.7°. This means that at least 12 per cent. of the potassium chloride had been taken out of solution.

The fact can also be shown in other ways though in not so obvious a manner. If we take distilled water and add successively equal amounts of KCl, each increment produces a slightly less increase of conductivity than the previous one. The reason is that as the solution becomes more concentrated the KCl becomes less dissociated ; to a less degree also increased ionic friction will contribute to the result. If, on the other hand, we add to the water first some 5 per cent. or so of gelatin and afterwards the successive doses of KCl the result is different. In a particular experiment 10 grams of gelatin were placed in 200 c.c. of distilled water, the gelatin having been previously washed fairly free from electrolytes. The conductivity of the water was raised to 26.9 gemmhos. When 2 c.c.  $\frac{N}{10}$  KCl were added and allowed to stand for 24 hours the conductivity was increased by 200 gemmhos,

Another 2 c.c. were added, and after 24 hours the conductivity was found increased by 208 gemmhos. That is, as I interpret it, a part of the first dose of KCl was taken up by the gelatin. It is impossible to compare directly these figures with those obtained on a similar experiment with distilled water without gelatin, since the proportion of water taken up in the swelling of the gelatin is unknown.

Again, if gelatin has the power of taking up electrolytes, it is to be expected that commercial gelatin and 'washed' gelatin would show a difference in their power of taking up more, since commercial gelatin has already a larger percentage. The following experiment was made to find out if this is so :—

*Experiment* : 12 grams (air-dry) of commercial and 'washed' gelatin were soaked for 20 hours in distilled water. The conductivity of the water was then found to be :—

Commercial gelatin : 553 gemmhos.

Washed       , : 7·7 gemmhos.

Two solutions (very dilute) of calcium sulphate and phosphate were made equal in conductivity to each of these extracts by adding, drop by drop, a saturated solution of these two substances in distilled water. Of these solutions a volume was taken equal to that of the water in two flasks in contact with the two kinds of gelatin, this was found to be (by weighing) 176 grams. Then to each of the four flasks 15 c.c. of the above-mentioned saturated solution of calcium sulphate and phosphate were added, and the increase of conductivity so produced was determined. If a part of the added electrolytes were taken up by the gelatin there should be a greater rise of conductivity in the cases of the watery solutions than in those where gelatin was present. The results actually obtained are rather difficult to interpret. To take first the 'washed' gelatin and the watery solution equal to it in conductivity. The rise of conductivity in the latter by the addition of 15 c.c. of the calcium sulphate solution was 163 gemmhos at 10°. Where the gelatin was present the first effect was a rise of 238 gemmhos, owing, no doubt, to the electrolytes not at once diffusing into the water contained in the swollen gelatin, this steadily diminished, and in about forty-five minutes had become 128 gemmhos. But then

the conductivity commenced to go up again, finally becoming 160 gemmhos. If, therefore, we take this last as the true value, the amount of electrolyte taken up by gelatin was very small, a difference of 3 gemmhos only between the two cases. If, on the other hand, we take the lowest value to which the conductivity fell, the difference is considerable, viz., 75 gemmhos.

In the case of the commercial gelatin and its equivalent calcium sulphate solution, the rise produced by the addition of 15 c.c. of saturated calcium sulphate and phosphate was 68 gemmhos in the watery solution and 56 gemmhos in that where the gelatin was present, a difference of 12 gemmhos. The addition of the electrolyte at first caused a rise of 106 gemmhos where the gelatin was present, it fell to 72 in one hour, but the final value was not reached for several hours, as opposed to the first case, where it was reached in two-and-a-half hours.

This experiment affords a certain amount of evidence that electrolytes are taken up by gelatin, but does not distinguish between adsorption and chemical combination. As we shall see later, the effect of heat shows it to be, in all probability, a case of the former.

### III. THE KINETICS OF ADSORPTION

#### 1. *Velocity of Reaction.*

Very little information is to be obtained as to the rate at which bodies are taken up by adsorbing substances. A few experiments that I have made on this question may therefore be of interest.

*Experiment:* 50 c.c. of 20 per cent. alcohol in test-tube lined with filter-paper. In thermostat at 39°. When warmed, 1 c.c. warmed 0.5 per cent. congo-red added, stirred, conductivity taken at intervals by pipette electrodes. The curve (Fig. 3) shows the time course of the diminution of conductivity. It will be noted that the curve is hyperbolic and that it arrives nearly at the asymptote in 10 minutes. At this temperature the velocity is considerable. The first part of the curve in all probability escaped observation, owing to the great initial velocity of the reaction as shown by the shape of the curve.

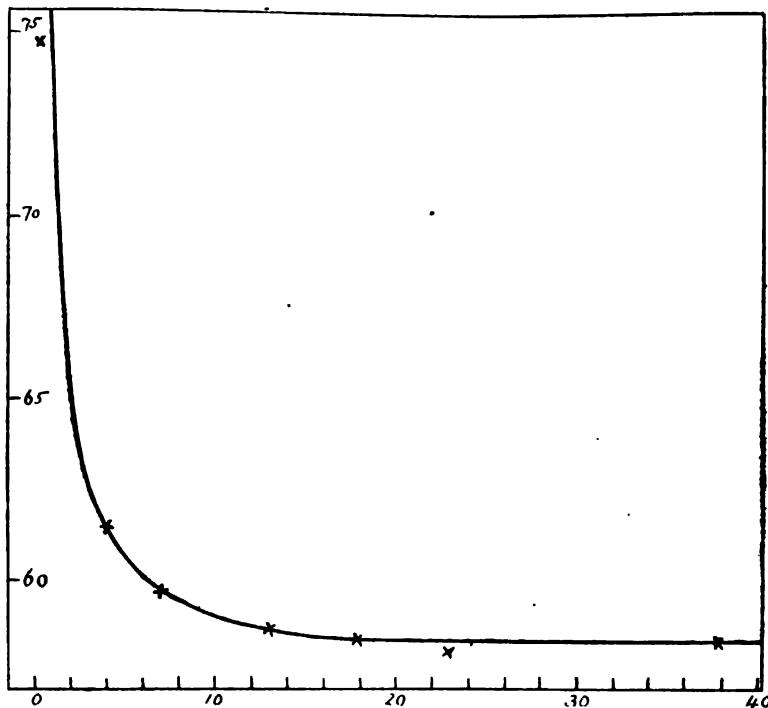


FIG. 3

In another experiment, in which the proportion of paper to dye was greater than in the previous one, equilibrium was reached in six minutes. So that the rate of change is proportional in the first place to the concentration of the adsorbing body. I have not enough data, however, to warrant any further discussion of this point. The reaction is one taking place in a heterogeneous system and its mathematical expression a complex one. It is probable that diffusion plays a considerable part as indicated by the results of the next experiment, which was made to obtain some idea of the temperature coefficient of the reaction-velocity. It was found that, for some reason or other—perhaps some action of the platinized electrodes on the dye—at low temperatures the electrical conductivity method did not give satisfactory results, so that the colorimetric method, though more laborious and less sensitive, was made use of.

*Experiment:* 50 c.c. of 0.005 per cent. congo-red in 5 per cent. alcohol in each of two flasks, a circle of filter-paper added to each, and

one kept at  $50^{\circ}\text{C}$ , the other at  $10^{\circ}\text{C}$ . After a certain time the solution was poured off and a new piece of paper and fresh dye solution, previously warmed, or cooled respectively, added and a different time of action allowed. The flasks were thoroughly shaken at frequent intervals to allow access of the solution to the whole surface of the paper. This shaking caused the separation of bits of paper which rendered it necessary to allow the solution to deposit before making colorimeter observations ; neglect of the precaution caused the loss of several of the earlier observations. Filtration is naturally inadmissible. The results were as follows, the colorimeter readings expressing the relative percentage of dye left in solution, the original solution = 100.

Duration of action	Colorimeter readings	
	$50^{\circ}$	$10^{\circ}$
5 minutes	95	circ. 98
8 "	90	...
20 "	85	...
30 "	...	90
40 "	80	...
100 "	77	70
200 "	...	60
5 hours	75	...
7 "	...	55
14 "	75	48
24 "	...	45

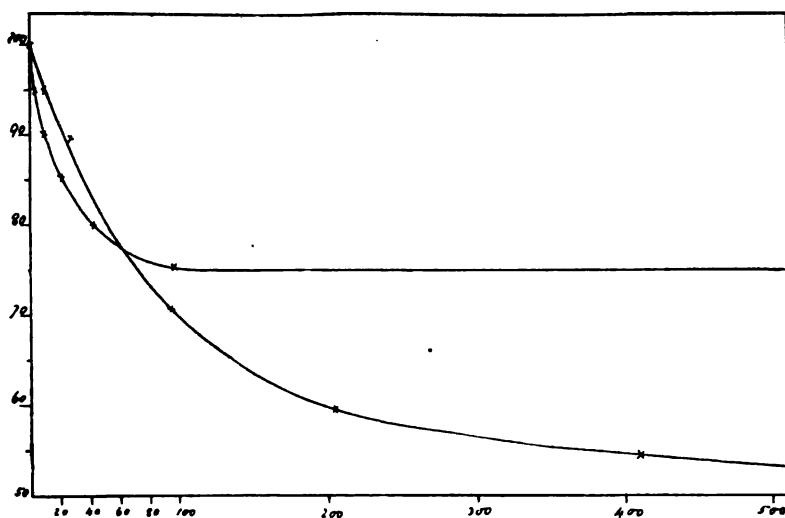


FIG. 4

The curves (Fig. 4) reproduce these two series up to seven hours. What we notice is that at the higher temperature the velocity is greater until the equilibrium position at this temperature is approached. The question of equilibrium will be discussed in the next section. In estimating the value of the temperature coefficient of the velocity, some uncertainty exists, since it will differ according to what stage of the reaction we take. If we take as criterion the time taken to reduce the concentration of the solution to a given percentage, say 90 per cent. at  $10^{\circ}$ , this time is about 2.5 times that at  $50^{\circ}$ , if we take 80 per cent. it is about 1.3 times. In either case, however, it is extraordinarily low ; taking the higher value at  $10^{\circ}$  it takes twenty-five minutes to become 90 per cent., and at  $50^{\circ}$  it takes ten minutes, a difference of fifteen minutes for  $40^{\circ}$  ; so that, assuming uniformity of increase for the total interval, the time taken at  $40^{\circ}$  would be only  $\frac{15}{4} = 3.6$  minutes more than at  $50^{\circ}$ , or ten minutes and 13.6 minutes respectively. This gives a temperature coefficient of the extremely low value of  $\frac{13.6}{10} = 1.36$ . In the table given by Van t' Hoff<sup>1</sup> there are only two values below this, viz., the dissociation of  $\text{PH}_3$  and  $\text{AsH}_3$ , probably depending on the high temperatures at which the observations were taken, since, as Van t' Hoff points out, the velocity-ratios for  $10^{\circ}$  usually diminish as the temperature rises. The value found in the present case indicates that the theory of Nernst<sup>2</sup> as to the part played by diffusion-processes in heterogeneous reactions applies here ; the corresponding value found by Brunner<sup>3</sup> for the dissolution of benzoic acid in water is, in fact, 1.5.

A few observations on the effect of alcohol and certain electrolytes on the velocity of reaction may be shortly referred to. Congo-red is the sodium salt of a substituted aromatic sulphonic acid, and is less soluble in alcohol than in water, so that it seems probable that more dye would be taken up from a solution containing alcohol than from a watery solution of the same concentration. This was found

1. Vorles. *über Theor. und Physik. Chemie.*, 2te Aufl. p. 225.
2. *Zeitsch. f. Physik. Chemie.*, XLVII, 1904, p. 52.
3. *Zeitsch. f. Physik. Chemie.*, XLVII, 1904, p. 62.

to be the case. Two solutions containing 0.005 per cent. of congo-red were taken, one in 50 per cent. alcohol, the other in water. From the former a circle of filter paper adsorbed at 14° in seventy minutes 45 per cent. of its contents, and from the watery solution only 30 per cent.

The action of a neutral electrolyte, sodium chloride, was similar, but more pronounced. From a solution containing 0.0042 per cent. dye and 2 per cent. NaCl, 75 per cent. was taken up in fifty minutes, and from a solution of the same concentration in dye, but without NaCl, only some 25 per cent. was taken up at the same time.

The action of strong acids is, of course, to set free the sulphonic acid from its compound, the free acid while more soluble in alcohol is less so in water, so that again we have a favouring effect on adsorption as follows :—Two flasks of the usual 0.005 per cent. solution of dye with each one circle of paper to 50 c.c. solution were prepared, one remained neutral, the other was acidified by the addition of one drop of 5 per cent. HCl. Though the solution became blue no precipitation of the colour-acid occurred in this weak concentration. After two hours at 13° to 14°, 35 per cent. was taken up from the acid solution and 12 per cent. from the neutral one. Before taking the colorimeter reading the acid solution was neutralized by a drop of ammonia.

It appears, then, that bodies which tend to diminish the solubility of congo-red favour its adsorption by the substances immersed in its solution.

The experiment with gelatin and calcium sulphate, previously related, shows that the rate of adsorption in that case is considerably greater than that of congo-red by paper, as would be expected from the greater mobility of the ions concerned. At 11° equilibrium was attained in less than two-and-a-half hours, whereas at the same temperature at least twenty-four hours was required for congo-red and paper.

## 2. *Equilibrium*

The most interesting fact under this head is the influence of temperature. As the curve (Fig. 4) shows, at the higher temperature,

less of the dye is contained in the paper and more in the solution. The curve (Fig. 5) shows the results of a few observations at various temperatures, ordinates being percentages in paper and abscissae temperatures. It will be noted that a straight line is formed. The values at the lower temperature are not quite so accurate as at the higher,

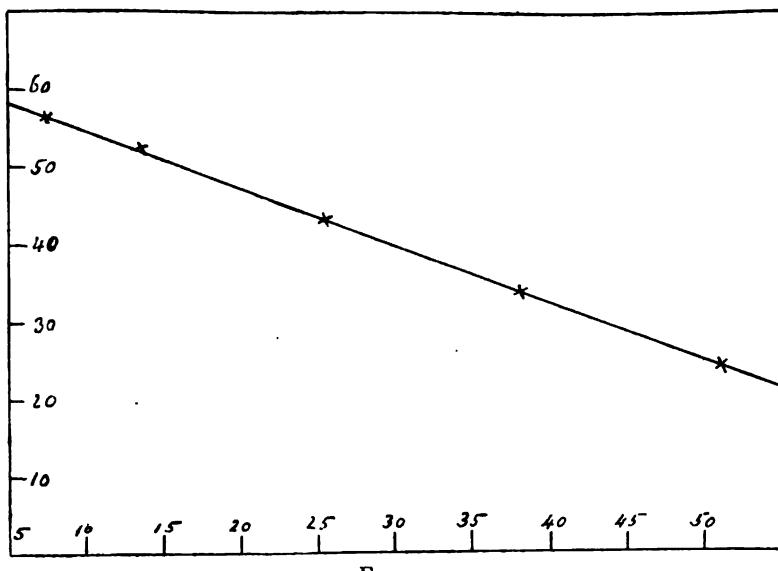


FIG. 5

since the former were obtained by allowing the flasks to stand in the laboratory, the temperature of which was not constant within  $2^{\circ}$  or  $3^{\circ}$  during the time necessary for equilibrium to establish itself. The upper values are accurate, being taken in a thermostat.

Attention has already been directed to the length of time necessary at low temperatures for the establishment of equilibrium in these dilute solutions. Ostwald also notes this fact.<sup>1</sup>

The dissociation of adsorption compounds by raising the temperature can also be shown in the case of gelatin and inorganic electrolytes, though complicated in this instance, by the passage from the state of hydrogel to that of hydrosol. There is, however, as I find, no sudden change in passing from the one state to the other as regards conductivity. If one warms a gelatin hydrogel from  $15^{\circ}$  to  $40^{\circ}$  determining

1. *Loc. cit.*, p. 1093.

conductivity at frequent intervals, the curve, which is in fact a straight line, shows no kink of any kind at the point where the hydrogel liquefies to a hydrosol.

The experiment previously described on the relations of gelatin to calcium sulphate was continued as follows :—The two flasks containing gelatin and calcium sulphate were warmed to  $53^{\circ}$ , the rise of conductivity in the case of the washed gelatin was 397.2 gemmhos, in the commercial gelatin 131.7 gemmhos. Now the point of interest is, whether this rise is greater than would be the case if no separation of additional electrolyte had taken place under the influence of heat. To decide this, solution of calcium sulphate was prepared, having at  $10^{\circ}$  the same conductivity as the solutions in contact with the two kinds of gelatin. These were then warmed to the same temperature as the gelatin. The rise of conductivity in the more dilute amounted to 376 gemmhos, and in that of the stronger to 144.6 gemmhos. There is therefore an increase in electrolytes in the case of the washed gelatin corresponding to a difference in conductivity of 21 gemmhos. It is to be remembered, moreover, that gelatin in the state of hydrosol has a slight effect similar to that of other non-electrolytes in diminishing the conductivity of solutions in which it is present. This is, no doubt, due to an increase of internal friction in some way or other, and amounts, according to determination I have made, to 3.4 per cent. diminution of conductivity for each 1 per cent. of the gelatin present. So that each of the values for the gelatin solutions should be increased by, approximately,  $3.4 \times 4.9$  per cent., the gelatin solution being 4.9 per cent. in concentration. This increases the two values to 463 and 153.6 gemmhos. The differences in favour of the gelatin come out now to be 87 gemmhos for the washed, and 90 gemmhos for the commercial sample. The closeness of these two numbers serves to strengthen the view that the electrolytes causing the increase were in some way separated off from the gelatin under the influence of heat.

It has been shown above that the conductivity of a solution of the ash from a given sample of gelatin is greater than that of the solution with which the gelatine is in equilibrium. I thought it interesting, therefore, to compare the ash of the particular 'washed'

gelatine of the above experiment, as regards its conductivity, when re-dissolved to original volume, with the conductivity of the gelatin solution at  $10^{\circ}$  and at  $53^{\circ}$ . The ash from 100 c.c. of the gelatin solution weighed 0.0262 gram, it was re-dissolved in 96.6 c.c. of distilled water (to allow for the volume of the gelatin), the whole of it not going into solution. Now the conductivity of the gelatin solution was found at  $6.2^{\circ}\text{C}$ . to be 141 gemmhos, that of the re-dissolved ash was 270 gemmhos. It is obvious that this is more than sufficient to account for the additional electrolytes split off on heating. It also confirms the view already expressed that inorganic electrolytes are held in some kind of combination by gelatin. Since they are also separated to a certain degree by warming, the combination in question is in all probability of the nature of adsorption.

The various substances which increase the velocity of adsorption also affect the final equilibrium position but in the opposite direction to that in which it is affected by heat. The action of neutral salts is the most interesting and of the greatest practical and theoretical importance. Sodium chloride has a very striking effect, even in very low concentration, as the following experiment shows :—

Solution of congo-red 0.004 per cent. containing various percentages of sodium chloride. After a circle of paper had lain in each for 2 hours the following colorimeter readings were obtained :

Percentage of NaCl	Percentage of dye left in solution	Percentage of NaCl	Percentage of dye left in solution
0	85	0.02	33
0.0005	77	0.1	25
0.001	71	0.2	16
0.005	50		

The effect of sodium chloride, then, appears to be proportional to its concentration but not in direct linear proportion. The fact that in so weak a concentration as 0.0005 per cent. an obvious effect is produced indicates that the action of inorganic electrolytes is rather to be brought into relation with the well-known effect of these bodies on typical colloids, rather than with the precipitating action of alcohol, which requires a much higher relative concentration in order to produce a comparable effect. In investigating this action of electrolytes

the phenomena met with, although of considerable interest, showed themselves to be of a very complex nature, so that it will be better to devote a special section to the discussion of them later on.

At first sight the action of heat in dissociating the adsorption compound, as above described, appears at variance with the common practice of histologists in staining their preparations. I find that the beneficial effect of heat in this case is probably due to the fact that a piece of paper, for example, dyed with congo-red and exposed to a temperature of 100° C. loses the power to a large extent of giving up the dye to water, in other words it is fixed more firmly than if merely stained at room temperature.

According to the well-known laws of Van't Hoff, since the compound of cellulose and congo-red is dissociated more and more as the temperature rises, the formation of the compound should be accompanied by evolution of heat. I have attempted in various ways to detect such a production of heat but have been unable to do so. The two methods used were : (1) Immersion of a Beckmann thermometer in the centre of a series of co-axial cylinders of filter paper in a large beaker filled with NaCl solution to facilitate adsorption. A test-tube of 1 per cent. solution of congo-red was also immersed in the solution, and when the whole was at the same temperature, the dye was mixed with the saline solution. No temperature change was to be observed, although by aid of a lens, the thermometer could be read to  $\frac{1}{1000}$ th of a degree ; (2) A disc of paper moist with NaCl solution was allowed to rest on the face of an ordinary bismuth-antimony thermopile contained in a vacuum-jacketed vessel and connected to a low-resistance d'Arsonval galvanometer. The vessel also contained a small tube of concentrated congo-red solution, closed at the bottom by a glass rod with indiarubber tube around its lower end. When the galvanometer was steady the glass rod was raised and the dye allowed to flow over the paper. No deflection indicating the production of heat was observed. It is to be noted, however, that at the temperature of these experiments, the process would not be very rapid, and it is possible that a slight heat production would be conducted away as fast as it was formed.

3. *Reversibility*

The question as to how far the process is a reversible one is of some theoretical interest. It has been already mentioned that if the temperature is raised up to  $100^{\circ}\text{C}.$ , a part of the dye is fixed in the paper, so that under certain conditions, the reversibility is not complete. At ordinary temperature it appears complete. If a circle of paper be dyed with congo-red, rinsed with water and then placed in water along with another similar circle of paper, it will be found that after some time the two pieces of paper will be of the same depth of colour. At the same time, as already pointed out at the beginning of this paper, to completely extract all the dye from a piece of paper needs an enormous number of repeated changes of water.

As regards temperature reversibility is apparently complete.

*Experiment:* Two flasks containing congo-red and filter-paper were placed, one at  $50^{\circ}$ , the other at  $11^{\circ}$ ; next day both flasks were placed in a thermostat at  $25^{\circ}$ , when equilibrium was attained after about 30 hours, the colorimeter readings were :

$$\begin{array}{ll} \text{From } 50^{\circ} & - 53\% \\ \text{, } 11^{\circ} & - 57\% \end{array}$$

Again, if sufficient time be allowed for equilibrium to be reached, a preparation may be alternately warmed and cooled, and nearly the same readings obtained at the same temperature. It appears, however, that after several days contact the dye becomes partly fixed. For example, in one case the reading at  $38^{\circ}$  was 66 per cent., it was then cooled to  $13^{\circ}$  with a reading of 48 per cent.; on again warming to  $38^{\circ}$ , 50 per cent. only was reached, and on cooling again to  $13^{\circ}$ , after two days the value became 25 per cent.

Gelatin, also, shows fairly complete heat reversibility as regards its adsorbed electrolytes. At  $11^{\circ}\text{C}$ . a particular hydrogel had a specific conductivity of 162 gemmhos, after being heated to  $53^{\circ}$  and again cooled to  $11^{\circ}\text{C}$ . its conductivity was 157.8 gemmhos. Another preparation containing more electrolytes had an initial conductivity of 609 gemmhos, after heating to  $55.5^{\circ}$  and cooling again to  $11^{\circ}$ , it became 542 gemmhos, or less than its original conductivity. Perhaps in the first measurement equilibrium was not completely attained.

When adsorption has taken place under the action of electrolytes a very considerable degree of fixation occurs, so that the dye is not given up again to water, or only to a very slight degree.

*Experiment:* Two similar pieces of paper dyed to same depth of colour, one in solution of  $\text{NaCl}$  0.02 per cent., the other in distilled water. Of course the latter solution was more concentrated as regards the dye. These pieces were rinsed in water, pressed between blotting-paper, and then placed in equal volumes of distilled water along with another piece of paper each. After 24 hours the one which had been dyed in distilled water had given up a considerable amount of pigment to the water, and the two pieces of paper were very nearly equal in depth of tint. The paper dyed in the presence of  $\text{NaCl}$  gave up no colour to be detected by the eye, although the second piece of paper was faintly pink, showing a slight extraction from the dyed piece.

#### IV. THE ACTION OF ELECTROLYTES

When we consider that a solution containing only 0.0005 per cent. of  $\text{NaCl}$  has a distinct effect in augmenting adsorption of congo-red by paper, viz., 23 per cent. taken up as against 16 per cent. from distilled water, it is plain that the phenomenon is not of the nature of a 'salting-out,' comparable to the precipitation say of egg-albumen by ammonium sulphate. The precipitation of such solutions as those of the colloidal metals and hydroxides is rather suggested. Now, there is considerable evidence that a large number of the anilin dyes exist in watery solutions in a colloidal form. Congo-red, having a molecular weight of nearly 700, would be expected to be one of these. Its solutions, in fact, do not diffuse through Schleicher and Schüll's parchment-paper thimbles, although they do so, very slowly, through ordinary parchment-paper. According to Michaelis<sup>1</sup> under the ultra-microscope they are heterogeneous, being resolvable into sub-microscopic particles. In the electric field the dye migrates to the anode, so that the particles are negatively charged. It is, however, somewhat difficult to make oneself certain of this fact on account of

1. *Deutsche Med. Wochenschrift*, 1904, No. 42.

the electrolytic decomposition. Colloids do behave as electrolytes, as is well-known<sup>1</sup>, and I have made one rough determination, by Whetham's boundary method, of the velocity of the coloured ion in congo-red solution. At 13° the boundary, which was not very sharp, moved 11 mm. in one hour under a potential fall of 3 volts per cm. Hardy<sup>2</sup> finds for globulin about 7 mm. in the same time, and for methylene-blue considerably more, about 40 mm. The electrical conductivity of congo-red solutions is comparable with that of inorganic electrolytes, a  $\frac{M}{100}$  solution at 40° having a specific conductivity of 5600 gemmhos. I do not lay any stress on the absolute value of this measurement since the preparation was not specially purified, and possibly contained a small amount of inorganic salt.

On the whole, then, we may regard congo-red as being a negatively charged colloid. As such it would be specially sensitive to di- and tri-valent kations, and this is in fact the case.

All the experiments to be described in this section, except when otherwise stated, were made in the same way, viz., to 50 c.c. of the solution of electrolyte 10 c.c. of  $\frac{M}{1000}$  dye solution and one piece 12.5 cm. diam. of Schleicher and Schüll's extracted filter paper added. After about 24 hours the amount of dye taken up by the paper was estimated by taking the colorimetric value of the dye left in solution.

It is easily seen at once that calcium salts are much more active in promoting adsorption than those of the monovalent alkali metals, e.g., the amount taken up from a solution containing  $\frac{M}{500}$   $\text{CaSO}_4$  was 85 per cent., from a  $\frac{M}{200}$  KCl 67 per cent.

Considering this fact the effect of tap-water is not surprising, since New River water contains an equivalent in  $\text{Ca}^{++}$  ions of about equal amount to the content therein of  $\frac{M}{300}$   $\text{CaSO}_4$ . From tap-water 85 per cent. was adsorbed in one experiment, and from distilled water only 27 per cent.<sup>3</sup> Moreover, these facts show that in order to obtain any reliable data, special care must be taken as to the purity of the paper used. As an illustration, I give the following experiment :

1. Hardy, *Journal of Physiology*, XXXIII, 1905, p. 292.

2. *Loc. cit.*, p. 291 and p. 289.

3. The absolute amounts in different experiments can only be compared by taking their ratio to the amount taken up from distilled water in each case, since the temperature was not the same in all.

Equal weights of the following samples of filter-paper were placed in 50 c.c. distilled water with 10 c.c.  $\frac{M}{1000}$  dye as usual. The amounts taken up by each are given :

1.	Dreverhof's ordinary, No. 333	...	...	95 %
2.	"	washed in distilled water		59 %
3.	Dreverhof's extracted paper	...	...	46 %
4.	"	washed	...	46 %
5.	Schleicher and Schull's extracted paper	...		46 %

I may mention here that Dreverhof's extracted paper turns blue when a drop of dilute congo-red is placed on it, this does not happen with Schleicher and Schöll's, nor with Swedish paper. As the above experiment shows this slight acidity does not seem to affect its adsorbing power, and as will be seen later  $H^+$  ions have comparatively little favouring effect.

The result of this experiment seems to suggest the possibility, that if one could obtain a complete absence of electrolytes no adsorption would take place.

As regards trivalent kations I have only tested aluminium sulphate, but as even in a concentration of  $\frac{M}{1000}$  precipitation took place the result was valueless.

It is scarcely necessary, perhaps, to remark that if the dye is precipitated by the electrolyte no adsorption by the paper takes place, the large particles merely float about in the liquid. What is needed is the local concentration of electrolyte on the surfaces of the adsorbing solids.<sup>1</sup>

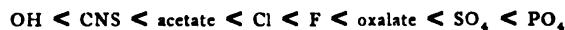
Although the greater favouring power of divalent as compared with monovalent kations is easy to demonstrate the order in each of these classes is difficult to decide since the action of each member is so nearly the same as that of the others. From a number of experiments the following seems to be the order :



1. Notwithstanding the fact that actual precipitation must not take place in these experiments the addition of electrolytes to congo-red, for example, causes an increase in the size of the colloidal particles even when no actual precipitation occurs ; so that the solution is on its way to precipitation even when this does not actually occur. The specimen of congo-red used in all my experiments showed the Tyndall phenomena very faintly in solution in distilled water ; but when NaCl was added the beam of light became much more distinct and the light reflected from it at right angles was polarized. When  $CaCl_2$  was added in sufficient amount to slowly precipitate the dye (about  $\frac{M}{100}$ ) the polarization ceased as the particles became larger than the mean move-length of light.

This is very nearly the same as that found by Pauli<sup>1</sup> in the case of precipitation of proteins.

It is a matter of some interest to see whether in this case of adsorption the anions have a retarding action like that shown by Pauli in the above-mentioned experiments. That there is some action of this kind can I think be shown by consideration of the comparative action of chloride and sulphate of the same metal, say potassium. The difficulty lies in the fact of the great preponderance of the opposite action of the kation, probably on account of the negative charge of the congo-red. If we take equimolecular solutions of  $KCl$  and  $K_2SO_4$ , we find their effect almost exactly the same within limits of experimental error. Now there are, in the dilute solutions employed, very nearly twice the number of kations in the  $K_2SO_4$  than in that of the  $KCl$ , necessarily, therefore, the  $SO_4$  ion has a greater retarding power than the  $Cl$  ion. In determining the order of the anions in this respect the same difficulty presents itself, as in the case of the opposite effect of the kations, but in a more marked degree. The approximate order as regards power of inhibition is the following:—



The results of these experiments will be best shown in a table analogous to those given by Pauli. The numbers give the percentage amount of dye adsorbed at room temperature under the influence of the body formed by the combination of kation and anion shown vertically above and horizontally at the left-hand side of each number, and in  $\frac{m}{200}$  concentration.

CONGO RED							
	H	Li	K	Na	$NH_4$	Mg	Ca
OH	26	58	...	68	79 ( $\frac{m}{12}$ )	...	89
CNS	...	...	...	...	64	86	...
Acetate	...	...	58	70	81	...	...
Cl	...	...	70	75	72	...	91
F	...	...	...	75	...	...	...
Oxalate	12	...	80	...	65	...	...
$SO_4$	4	71	74	78	78	91	> 100
$PO_4$	...	...	...	...	74	...	...

1. Hofmeister's Beiträge, III, p. 225; V, p. 27; VI, p. 233; and VII, p. 531.

There are some irregularities especially as regards  $\text{NH}_4$  salts. The value put against  $\text{CaSO}_4$  means that the concentration had to be taken at  $\frac{m}{500}$  to avoid precipitation, and at this strength the value was 84 per cent.

The action of  $\text{OH}^-$  and  $\text{H}^+$  ions needs a little further explanation since it seems to vary according to concentration in both cases. It is somewhat remarkable that in concentrations of  $\frac{m}{100}$   $\text{NaOH}$  and  $\text{NaCl}$  have almost exactly the same amount of favouring action ; in  $\frac{m}{1000}$  on the contrary, while  $\text{NaCl}$  has a distinct *favouring* action, viz., 58 per cent. adsorbed from  $\text{NaCl}$  as against 30 per cent. from water,  $\text{NaOH}$  has a very slight *inhibiting* action, viz., 25 per cent. against 26 per cent. from water.

In one experiment I took three solutions of equal content in  $\text{OH}^-$  ions, viz. :—

$\frac{m}{1000}$	$\text{NaOH}$	...	31 %
$\frac{m}{12}$	$\text{NH}_4\text{OH}$	...	79 %
$\frac{m}{100}$	$\text{Na}_2\text{CO}_3$	...	89 %

The amount adsorbed in presence of these is shown opposite each one.

I could not detect any influence of anilin-water on the process.

Sulphuric acid  $\frac{m}{2000}$  has a favouring action, 33 per cent. against 21 per cent. from water, while in  $\frac{m}{200}$  it has the opposite effect as the table shows. I am unable to suggest any explanation of these facts.

In connection with the well-known mutual precipitation of oppositely charged colloids<sup>1</sup> the behaviour of congo-red as a negative colloid is of interest. Colloidal platinum prepared by Bredig's method does not precipitate, but has a chemical action, turning the dye brown. This platinum sol is negatively charged so that precipitation would not be expected. On the other hand, according to Pauli<sup>2</sup> and other investigators, the precipitating action of salts of the heavy metals on egg-white is due to the presence in their solutions of metallic hydroxide in the colloidal form and presumably positively charged.

1. See Picton and Linder, *Journ. of Chem. Soc.*, 1892, page 148, etc., and W. Biltz, *Ber. d. Deutsch. Chem. Ges.*, 1904, Bd. XXXVII, page 1111.

2. Hofmeister's *Beiträge*, VI, page 257, 1905.

This being so, the powerful action of such salts on adsorption is not to be wondered at. In fact, I find  $\frac{m}{500}$   $ZnO_4$  to have an equal effect to that of  $\frac{m}{200}$  KCl.

This action of zinc sulphate gives the opportunity for testing by experiment whether in this case, as in Picton and Linder's<sup>1</sup> cases of precipitation of colloids by electrolytes, the precipitating ion is carried down with the colloid to become attached to the paper. Since the 'precipitating' agent in the adsorption of a negative colloid is the positively-charged cation, and the most convenient method of detecting a disappearance of an ion, is the use of a concentration battery in the manner of Nernst, it is plain that we cannot conveniently determine the question where salts of alkali metals are concerned. A concentration battery in  $Zn^{++}$  ions is, on the contrary, easily arranged. In the present case I took two vessels, each containing an amalgamated zinc electrode immersed in  $\frac{m}{500}$   $ZnSO_4$  solution and connected together by an inverted U-tube filled with the  $ZnSO_4$  solution. Such a battery being symmetrical has no E.M.F. A piece of filter paper was now placed in one of the vessels, the E.M.F. began to rise and attained a value nearly equal to what it would be by the Nernst formula, if all the  $Zn^+$  ions had disappeared from this vessel, viz., 0.0257 volt. This effect was, in all probability, due to adsorption by the paper. On now adding 5 c.c. of 1 per cent. congo-red in  $\frac{m}{500}$   $ZnSO_4$ , the E.M.F. went down again, due to the addition of more  $Zn^+$  ions. Presently, as the dye became adsorbed, the E.M.F. commenced to rise again and when plotted on squared paper showed the usual form of the adsorption-curve. As far as it goes, then, the experiment shows a diminution in concentration of  $Zn^{++}$  ions. But there are two circumstances which deprive it of much value. In the first place I found that next day the E.M.F. of the battery had risen to 0.094 volt, a value above that possible by removal of all the  $Zn^{++}$  ions; there must have been, therefore, some secondary process set up by the congo-red. In the second place the possibility must be taken into account, that when congo-red and zinc-sulphate are mixed, a zinc-salt of the congo-red may be formed by double decomposition which might not be completely dissociated, this

1. *Loc. cit.*

salt might be the actual body adsorbed by the paper. Considering the great dilution of the solution, however, I do not think the latter objection is very serious.

Another property of colloids is that of being protected from precipitation of electrolytes when a small amount of a stable colloid, such as gelatin, is present. As pointed out by Zsigmondy,<sup>1</sup> the fact was known to Faraday, although its meaning was, of course, not understood at the time. The following experiment shows that gelatin also protects congo-red from the action of an electrolyte :—

- A. 50 c.c. H<sub>2</sub>O + 1 c.c. 0.5% dye
- B. 50 c.c. 0.05% gelatin + 1 c.c. 0.5% dye
- C. 50 c.c. 0.038% NaCl + " "
- D. 50 c.c. 0.038% NaCl containing 0.05% gelatin + 1 c.c. 0.5% dye

Adsorbed in three hours :

A. 30 %	...	C. 74 %
B. 31 %	...	D. 34 %

It will be seen that NaCl is practically without effect in presence of gelatin.

Egg-albumin has a similar effect, but less marked, probably on account of its content in electrolytes. I did not subject it to dialysis.

Gelatin is stated by Victor Henri<sup>2</sup> to be a negative colloid ; serum-albumin has recently been shown by Pauli<sup>3</sup> to be positive in acid solution, and negative in alkaline solution. It was of interest therefore, to see whether any difference in the protective action of egg-albumin was to be noted in the two instances. The result showed that electro-positive albumin *increased* the action of  $\text{CaSO}_4$ , while electro-negative albumin *diminished* it :—

- A. Albumin 0.04% +  $\text{CaSO}_4 \frac{m}{500}$  +  $\text{H}_2\text{SO}_4 \frac{m}{1000}$
- B.  $\text{CaSO}_4 \frac{m}{500}$  +  $\text{H}_2\text{SO}_4 \frac{m}{1000}$   
+ dye + paper as usual

Adsorbed :

- A. > 80% (partially p<sup>td.</sup>)
- B. 35%

1. *Loc. cit.*, p. 65, footnote.

2. *Rev. Gen. des Sciences*, 1905, p. 641.

3. Hofmeister's *Beitrage*, VII, p. 535 and p. 536.

C. Albumin 0.04 % +  $\text{CaSO}_4 \frac{\text{m}}{500}$  +  $\text{NaOH} \frac{\text{m}}{1000}$

D.  $\text{CaSO}_4 \frac{\text{m}}{500}$  +  $\text{NaOH} \frac{\text{m}}{1000}$   
+ dye + paper.

Adsorbed :

C. 75 %  
D. 98 %

It is difficult to compare with these the action of the neutral albumin since acid and alkali have themselves an influence. But it appears that the sign of the charge on the stable colloid determines the nature of its action, although the particular albumin in this experiment had also a protective action in neutral reaction. I am unable to state whether it was naturally electronegative or otherwise.

Other electro-negative dyes, such as anilin-blue and nigrosin, under the action of electrolytes, give similar results to congo-red. The colloidal condition of the solution is, however, apparently a *sine qua non*. I could not detect any influence of electrolytes on picric acid, although it is difficult to observe a small difference, because of the comparatively feeble colouring power of this dye. I compared the depth of tint of the two solutions, one with electrolyte, the other without, in both of which the same amount of paper had been immersed, by placing them in two cells of equal thickness in front of a piece of plantinotype paper and exposed to light. No difference could be detected in the depth of tint on development of the paper, although, as is well known, this paper shows very small differences of tone.

Scharlach R. is interesting, because it is a so-called 'indifferent' dye, that is it does not form salts. It is insoluble in water; but as Michaelis<sup>1</sup> points out, when an alcoholic solution is mixed with five to six times its volume of water, it is not precipitated, but becomes a colloidal solution. In this state it behaves towards electrolytes like a negative colloid, and, in fact, in the electric field, as I find, it wanders to the anode.

Rosolic acid shows similar properties.

1. *Deutsche, Med. Wochensch.*, 1904, No. 42.

In order to save space, I have put together in a table at the end of this paper a number of facts relating to various dyes in common use which have a bearing on the question under discussion.

The only other point I will refer to as regards the negative dyes is that eosin, although its colloidal properties are doubtful (see table), shows a much greater sensitiveness to the anion than the dyes hitherto mentioned, alkali in this case causes the taking up of *less* colour by paper than is taken up from water. We found in the previous cases that the action of the  $\text{OH}^-$  ion was overpowered by the opposite action of the kation ( $\text{Na}^+$ ).

We turn now to the electro-positive dyes like toluidin-blue. It would be expected that in this instance the anions would have a favouring action, the kations the reverse. This is, indeed, the case, but like the negative dyes the effect of the kation is predominant, so that the result of adding any electrolytes, except alkalies, is to *lessen* adsorption. In the absence of electrolytes, as a rule, more dye is taken up by the paper than from the solution of an electro-negative dye. This would appear to be at variance with the general use of congo-red to dye cotton, but it is to be remembered that in practice there is always sufficient electrolyte present to reverse the relative behaviour of negative and positive dyes as shown when dissolved in pure distilled water.

For details as to various positive dyes see the table.

Methylene blue precipitates the electro-negative colloidal platinum.

I have not been able to detect any marked protective action of stable colloids in the case of electro-positive dyes.

In the circumstance that both the + and - dyes are more sensitive to kations than to anions they behave like albumin solutions to salts of the heavy metals as shown by Pauli.<sup>1</sup>

On reference to the table at the end of this paper it will be noticed that in all the cases investigated where the dye is in the form of a salt with an inorganic base or acid, as the case may be, the sign of the charge of the colloid is determined by the organic constituent of

1. Hofmeister's *Beiträge*, VI, pp. 233 to 249.

the salt. In this respect they differ from the globulin of Hardy and the serum-albumin of Pauli, which in acid or alkaline solutions assume the sign of the charge of the  $\text{H}^+$  or  $\text{OH}^-$  ions respectively. They are in fact more like certain complex ions, such as the ferrocyan ion,  $\text{Fe}(\text{CN})_6$ , which, although containing an iron atom is, nevertheless, electro-negative.

I have been unable, for want of time, to test the behaviour of more than a few typical dyes of the two classes, but it appears that there is considerable difference of degree in the sensitiveness to electrolytes. This property indeed seems to depend on the degree of colloidality of their solution and the amount of their electric charge. Each individual dye requires separate investigation.

As to the explanation of the action of electrolytes, I think the clue is given by the following considerations. V. Henri and Larguier des Bancels,<sup>1</sup> in their work on colloids, observed that gelatin, as hydrogel, when immersed in a solution of anilin-blue or congo-red in distilled water took up little or none of the pigment, and give as the reason for this that both the bodies are electro-negative colloids and therefore mutually repel one another. If, on the contrary, a solution of a bivalent metal, such as barium nitrate, is added the gelatin becomes deeply stained. The negative charge of the anilin-blue is neutralized by the positive barium ions so that it can now freely attach itself to the gelatin. This case then resolves itself into one of the mutual precipitation of colloids, and the only doubtful point about the explanation is whether the negatively-charged particles of the gelatin hydrosol may be regarded as retaining their charge when in the form of gelatine. There seems no *a priori* reason why this should not be so, and there is also experimental evidence in favour of it. Picton and Linder<sup>2</sup> in referring to the adsorption affinity of the hydrogels of ferric hydroxide and arsenious sulphide for anilin dyes state that these hydrogels 'retain the same selective affinity for the dyes which will coagulate them' as they possessed as hydrosols, viz., 'the hydrogel of ferric hydroxide for anilin-blue, that of arsenious

1. *C.R. Soc. de Biologie*, LIX, p. 132, 1905.

2. *Journ. Chem. Soc.*, 88, 1905, p. 1934.

sulphide for methyl-violet. We regard this fact as evidence that the granular aggregates in these hydrogels still retain some charge. In other words, the difference of potential existing between the molecular aggregates and the field in the hydrosol state is not destroyed by coagulants, but only reduced to a point at which the forces of cohesion are just able to overpower the forces of repulsion brought into play by such difference of potential.'

It will readily be seen that if paper assumes a negative charge when immersed in water the phenomena I have described above fall into line with the behaviour of electrically charged colloids. We notice that of a negative colloid like congo-ied the less is taken up by paper the more perfect the freedom of the water from electrolytes, there is mutual repulsion until the dye is discharged by a kation. On the other hand, the opposite holds as regards the positive dyes, there is attraction of the negative paper for the positive dye. The kation in this case probably acts by discharging the paper, being attracted thereto by its opposite charge.

Prof. Donnan, to whom I propounded the question, refers me to the work of Coehn<sup>1</sup>, who found that when various non-conducting bodies are immersed in fluids of different dielectric-constants they assume a positive or negative charge according as their own dielectric-constants are higher or lower than the fluid with which they are in contact. For instance, glass (5.6) is negative in water (80) or alcohol (26), whereas in turpentine (2.2) it is positive. Now, paper according to Thwing<sup>2</sup> has a dielectric-constant of 2.82, and would therefore be electrically negative to water.

Moreover, Quincke<sup>3</sup>, in the course of his investigations on electrical endosmosis found that, as a rule, all non-conducting bodies in water took on a negative charge. Among the substances tested by him were cotton-wool and silk ; particles of these in water exposed to an electric field wandered to the anode and were therefore negatively charged.

1. *Wied. Ann.*, 64, page 217, 1898.

2. *Zeitsch. f. Physik. Chemie.*, XIV, page 292, 1894.

3. *Pogg. Ann.* 113, page 583, 1861.

The conclusion is, I think, justified that paper takes up very little electro-negative dye because it is itself negatively charged, and that when the dye is discharged by the addition of an electro positive colloid or a kation, there is no longer the same mutual repulsion between the dye and paper.

It would be of interest to test the behaviour of paper in turpentine, in which it would be electro-positive, if one could obtain an electro-negative colloidal dye in solution in turpentine.

This interpretation is confirmed by the results of experiments on silk :—

*Experiment* : From a watery solution of congo-red a piece of paper took up 26 per cent., a piece of silk (well washed), of the same weight took up only 5 per cent. From a  $\frac{m}{200}$   $\text{MgSO}_4$  solution, paper took up 91 per cent. and silk 98 per cent. From toluidin-blue in water, paper took up 85 per cent. Silk rapidly took up the whole, so that I added another double amount of dye solution, the colorimeter reading thus showed that 67 per cent. of the whole, that is  $67 \times 3 = 201$  as compared with 85 in the case of paper was taken up. The action of neutral-salts on the adsorption of positive dyes by silk was of the opposite sign to their effect on paper. For example :—

Paper : From water	...	...	85 % adsorbed
", $\frac{m}{200}$ $\text{MgSO}_4$	...	51 %	"

Silk : From water (concentration of dye three times that in case of paper)	67 % adsorbed
", $\frac{m}{200}$ $\text{MgSO}_4$	91 % "

The results may be explained by the consideration that silk, owing to its lower dielectric constant than that of paper, would no doubt have a higher negative charge, which would make it less accessible to negatively-charged dyes, but more so to positively-charged dyes. I am unable to state why the action of electrolytes on the positive dye is opposite in the two cases of silk and paper, unless that for some reason silk is relatively more sensitive to the adjuvant action of the anion. If this were so, however, one would expect a more marked

inhibitory effect of the anion in the case of electro-negative dyes and silk, whereas we see that the action of  $MgSO_4$  is rather greater in the case of silk than in that of paper.

The troublesome stain which appears on the surface of glass containing electro-positive dyes is no doubt due to the negative charge of the glass. This film is so adherent that water will not remove it, and I have found it necessary, when working with these dyes, to rinse all flasks used with concentrated nitric acid, in order to obtain concordant results.

Another fact receives its explanation from the negative charge of paper, viz., that from alcohol more negative dye is taken up than from water, in one experiment :

From 50 % alcohol - 65 % taken up  
 " water - 53 % "

Since alcohol has a lower dielectric-constant than water it would naturally be expected, since the negative charge of paper is due to the difference between its dielectric-constant and that of the fluid in which it is immersed, that the negative charge would be greater the greater this difference is, and therefore in alcohol the charge would be less than in water and the attachment of a negative dye less difficult. On the other hand, less + dye is taken up in the presence of alcohol, thus :

From 50 % alcohol - 5 % taken up  
 " water - 80 % "

Finally, I may refer to the action of acid in preventing the favouring action of electrolytes on adsorption of a negative dye, for example :

#### CONGO-RED

From water	...	...	...	27 % adsorbed
" $\frac{m}{200}$ NaCl	...	...	...	75 % "
" $\frac{m}{200}$ NaCl + $\frac{m}{2000}$ $H_2SO_4$	...	...	31 % "	

This result is probably to be explained by the fact that in the presence of sulphuric acid the colour-acid is set free from congo-red. This colour-acid is, no doubt, more strongly electro-negative

than the salt, so that the amount of kation which would suffice to discharge the salt would be insufficient to discharge the acid, it would therefore be comparatively ineffective in promoting adsorption.

In view of the theory set out in the preceding paragraphs it will be of interest to give two curves showing the form of the relation between the concentration of the kation and its effect on congo-red. The ordinates in Fig. 6 represent the percentages of congo-red taken up from a  $\frac{m}{6000}$  solution containing electrolyte of the concentration given by the abscissae. The upper curve is that of  $\text{CaSO}_4$ , the lower curve that of  $\text{KCl}$ .

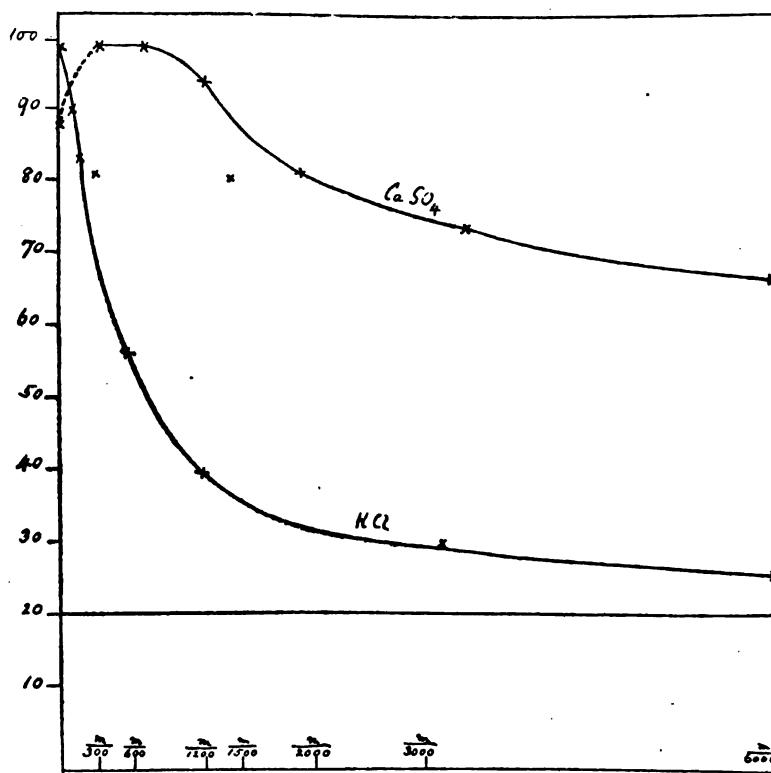


FIG. 6

The commencement of the  $\text{CaSO}_4$  curve is irregular, owing to partial precipitation of the dye having taken place. The inclination to an S-shape at the beginning of the  $\text{KCl}$  curve is, probably, also an indication of incipient precipitation. The extreme sensitiveness of

congo-red to electrolytes is also shown by the curves ; even at the low concentration of  $\frac{m}{6000}$  of the electrolyte they do not fall to the level of the amount taken up from the particular sample of water used, which amount is represented by the horizontal line with an ordinate value of 20 per cent. It is noticeable, on the contrary, that the curves tend to become asymptotes. I am not prepared to give any interpretation of the form of these curves.

## V. CONDITIONS OF DISSOCIATION OF ADSORPTION COMPOUNDS

Recent research has brought to light the important part played in vital phenomena by the mutual relations of colloids to electrolytes and to other colloids. Since these relations are apparently those of adsorption, it is of some importance to know what are the conditions under which this association is broken down.

### 1. *Temperature*

We have already seen that dissociation is favoured by rise of temperature, and that, on the other hand, if suddenly exposed to the temperature of boiling water, a dyed piece of paper becomes to a certain degree fixed in its condition, so that the dye can only with difficulty be extracted by water.

### 2. *Precipitation*

It was noticed by Cramer and Swale Vincent<sup>1</sup> that the inorganic constituents of various organic bodies are set free when these bodies are acted upon by precipitants of bases. I have observed the same fact in the case of the precipitation of gelatin by tannin.

*Experiment* : A 15 per cent. solution of gelatin had a specific conductivity of 1570 gemmhos at 38.5. 20 c.c. of this solution were mixed with 90 c.c. water and 10 c.c. of 10 per cent. tannin solution, filtered, and 100 c.c. filtrate concentrated to 20 c.c. The conductivity of this was now 2750 gemmhos. This 20 c.c. would represent  $\frac{100}{120}$  of the first, so that the conductivity should be multiplied by  $\frac{120}{100} = \frac{120}{100} = 3300$  gemmhos, that is, the conductivity was doubled by the action of

1. *Journ. of Physiology*, XXX, p. 150, 1903.

tannin. The amount of tannin added was intentionally less than that required for complete precipitation, so that the filtrate contained a little unaltered gelatin, but no tannin.

### 3. Action of Chloroform

Moore and Roaf<sup>1</sup> have shown that electrolytes are set free in blood by the action of chloroform.

### 4. Excitation, Injury, and Death

Macdonald<sup>2</sup> found that, as a result of injury, potassium salts are set free in the axis-cylinders of nerve fibres. Under certain conditions these salts may be taken up again by the colloids, and Macdonald suggests a theory of excitation and inhibition on this basis.

Hoeber<sup>3</sup> finds that the excitability, the capacity of staining, and the colloidal consistency of nerve vary concurrently.

Wakelin Barratt<sup>4</sup> finds that chlorine ions are set free when unicellular organisms are injured or killed.

Brailsford-Robertson<sup>5</sup>, as the result of his work on the phenomena of the heart-beat, comes to the conclusion that these phenomena are such as would be expected if there were a periodic driving out by anions of kations from combination with proteid.

Howell,<sup>6</sup> having found that the vagus cannot exert its action on the heart-muscle in the absence of potassium ions, brings forward the hypothesis that the inhibition produced by the vagus is due to splitting off potassium from combination in the muscle.

In order to see whether there is any evidence of a sudden separation of electrolytes at the moment of death, I have made the following experiment :

The skins of two frogs were immersed in isotonic cane-sugar solution. The electrical resistance of the solution was measured, after soaking for some time, and found to be at 11° C., 6407 ohms. The solution containing the skins was then slowly warmed and the resistance determined at frequent intervals up to 56° C. The

1. *Proc. Roy. Soc.*, 73, p. 382, 1904, and 77 B, p. 98, 1906.
2. *Thompson-Tate Lab. Report*, Vol. 4, p. 213, 1902.
3. *Centralb. f. Physiologie*, XIX, page 390, 1905.
4. *Zeitsch. f. Allgem. Physiologie*, V, page 33, 1905.
5. *Pfluger's Archiv*, 110, page 623, 1905.
6. *American Journ. of Physiology*, XV, page 291, 1905.

values when plotted on squared paper formed a regular curve, somewhat concave towards the axis of abscissae. There was no indication of any kink in the curve except for one determination at  $16^{\circ}$ , which was obviously an experimental error. That electrolytes were given off is shown by the fact that the diminution of resistance was considerably greater than would have been the case if the diminution depended only on the effect of the rise of temperature on electrolytes present at the beginning. The temperature-coefficient was, indeed,  $+14$  per cent. per degree, instead of, at the most,  $2.75$  per cent., according to the determinations of Arrhenius. The separation of electrolytes also showed itself to be irreversible, since cooling down to  $11^{\circ}$  C., the resistance only increased to 4128 w. The shape of the curve shows that the rate of separation was greater at the lower than at the higher temperatures, but, as already remarked, there was no point on the curve to indicate that death occurred. It is obvious that the results may also be accounted for by gradual destruction of the impermeability of the living cell for most inorganic salts, but, if so, this process must also be a gradual one.

To compare with this experiment I made a similar one with egg-white, raising the temperature to  $95^{\circ}$  C. In this case the result was opposite to the above, viz., there was evidence of taking up of electrolytes. The physical state of the coagulated egg-white is, however, so different from that of the fresh substance that it is difficult to interpret the meaning of the observation.

##### 5. *Coagulation of blood.*

It was found by E. G. Martin<sup>1</sup> that  $Ca^{++}$  ions have a marked stimulating influence in causing freshly isolated strips of the tortoise ventricle to commence beating spontaneously. This effect is shown by serum, but not by uncoagulated plasma. It appears, thus, that  $Ca^{+}$  ions are set free during the process of clotting, so that there should be an increase of electrical conductivity in the process. Robert T. Frank<sup>2</sup> was unable to detect any change of this kind. I have, on the contrary, succeeded in showing that there is such a change, not in

1. *Amer. Journ. of Physiology*, XI, p. 117, 1904.

2. *Amer. Journ. of Physiology*, XIV, p. 466, 1905.

the sense of an *increase* but a *decrease*, so that there is a disappearance of ions. This result is, I think, what would be expected from our knowledge of the relation of calcium to clotting, and the observations of E. G. Martin must be explained in another way. My experiment was made on fowl's blood. As shown by Delezenne, if kept from contact with the tissues this blood remains unclotted for a long time. It was collected from the external jugular vein and placed in a U tube furnished with an electrode in each limb and immersed in a water-bath at  $10.2^{\circ}$  C. When the temperature had become constant the specific conductivity of the blood was 5382 gemmhos. A small piece of muscle from the fowl was then dropped into one side of the tube. The conductivity steadily fell to 4360 gemmhos, which value was attained in 40 minutes; no doubt the process was completed earlier than this, but it was not wished to disturb the apparatus until it was to be expected that clotting had occurred. The diminution of conductivity therefore amounted to 18.5 per cent. of the original value.

#### 6. Action of enzymes

I do not purpose here to enter into this subject in any detail, since it will be dealt with fully in a forthcoming paper. There is a certain amount of evidence that the splitting-off of inorganic constituents contributes to the rise of electrical conductivity observed in many cases, but the direct proof was found to be of considerable difficulty.

In the action of rennet on milk, I find a slight *increase* of conductivity. This is merely an additional fact showing the nature of this process to be quite different from that of clotting of blood. In all probability rennet-action is only an expression of pepsin-action in neutral or faintly alkaline medium.<sup>1</sup>

#### VI. SPECIFIC ADSORPTION

The phenomena of adsorption show in this respect an approximation to true chemical combination.

It was shown by Schönbein<sup>2</sup> that when strips of filter-paper were immersed in solutions of various salts that the height to which the salts

1. Pawlow and Parastschuk. Hoppe-Seyler's *Zeitch.*, 42, p. 415, 1904.

2. Poggendorff, *Ann.*, 114, p. 275, 1861.

rose was less in the case of calcium and barium than in that of potassium or strontium, the calcium, etc., being more completely held by the paper. In the case of a solution of iodine in KI, while the latter rose nearly as far as water, the iodine was kept back in the lower third. These facts were explained by Schönbein as capillary attraction, but, as Ostwald remarks, there is no doubt that it is really an adsorption process that causes the separation of the bodies in question, capillarity only occasions the transport of the separated fractions.

These results of Schönbein have been extended and employed as a means of analysis by Goppelsroeder.<sup>1</sup>

It is well known to histologists how certain tissues take up particular dyes in preference to others. In certain cases, as for example, the differences between paper and silk, as to their respective behaviour towards congo-red and methylene blue, this is probably to be accounted for by the fact of the electrical charge in the two dyes being opposite in sign combined with the negative charge of the substance to be stained, as already pointed out.

Acid fuchsin is used in Van Gieson's stain to show connective-tissue. In view of this, I thought it of interest to try the behaviour of gelatin in sheet towards this dye and toluidin blue respectively. The same amount of gelatin was found to take up 72 per cent. of the acid-fuchsin and only 32 per cent. of the blue. It is possible here that the electrolytes of the gelatin were responsible for the difference, since they would increase the adsorption of the acid dye and diminish that of the basic dye. This possibility is excluded when we compare the amounts of congo-red and acid fuchsin, both electro-negative dyes, taken up by gelatin or filter-paper. The following are the results of an experiment:

Equimolecular concentrations of dye.

Equal weights of gelatin and filter-paper.

Filter-paper from Congo-red	...	31 %	
"    "    acid-fuchsin	...	32 %	
Gelatin	"    Congo-red	...	49 %
"    "    acid-fuchsin	...	95 %	

<sup>1.</sup> *Mitt. d. techn. Gewerbe Museums, Wien, 1899, and Studien über die Anwendung d. Capillaranalyse, Basel, 1904.*

Gelatin, then, has a specific adsorption affinity for acid fuchsin.

The preference exhibited by silk for methylene-blue over congo-red, previously described, may be explained in another way than by specific adsorption, viz., by the probable greater negative charge of silk.

That gelatin has a greater adsorption-affinity for calcium sulphate than it has for potassium chloride seems to be shown by the experiments on washing out electrolytes, given in the earlier part of this paper. It was found that  $KCl$  is more easily washed away than calcium sulphate.

## VII. THE APPLICATION OF THE FACTS OF ADSORPTION-PROCESSES TO VARIOUS PHENOMENA

The theoretical interest of adsorption-processes has been pointed out by Ostwald,<sup>1</sup> who suggests the possibility that a mechanical theory of chemical affinity may be developed on the basis of a complete study of these phenomena.

From a more practical standpoint, it will be found that they play a very important part in numerous processes. I may refer briefly to a few of these.

### 1. *The Soil*

The power of the soil in holding back soluble salts and other bodies is, no doubt, due to adsorption. By this means these bodies are prevented from being readily washed away by the rain.

### 2. *Purification of sewage*

In the filter-process of nitrification, it appears that the complex organic substances, which are inimical to the nitrifying organisms, are kept back by adsorption in the upper layers of the filter.<sup>2</sup>

### 3. *The ash-constituents of organic compounds*

I have already given evidence that these are present in an adsorbed form, and shown that the difficulty of removing the last fractions of inorganic electrolytes is adequately explained on this basis.

1. *Op. cit.*, p. 1098.

2. See Dr. Harriette Chick, *Proc. Roy. Soc.*, 77B, p. 247, 1906

It is scarcely necessary to give instances of this difficulty, and I will merely refer to three instances. Macallum<sup>1</sup> in searching for the cause of the silver reaction of tissues met with considerable difficulty in completely removing chlorides. Mellanby<sup>2</sup> states that there is a molecular combination between neutral salts and globulin. Lange<sup>3</sup> found that it was impossible to remove all ash-constituents from filter-paper by extraction with hydrochloric and hydrofluoric acids and washing.

#### 4. *Dyeing*

On the whole, I think it must be taken as the true explanation of this process that it is, in the main, an adsorption. The very slight diffusibility of most dyes through parchment-paper seems to me a considerable objection to Witt's theory of solid-solution. If the dye is dissolved in the paper it should be readily given off again to water on the opposite side in the same way as hydrogen passes through palladium in the experiments of Ramsay.<sup>4</sup> The theory to which my experiments lead is very much the same as that to which Picton and Linder have independently arrived.<sup>5</sup> According to these observers, there are two stages to be distinguished.

Stage I. The 'coagulation stage' in which single ionic interchange takes place between the 'fibre substance' (colloid) and the dye, resulting in the separation of insoluble dye derivatives retaining a feeble charge.

Stage II. The 'colour absorption' stage, in which coagula produced in stage I attract and retain the oppositely-charged particles of the dye substance.

My experiments show that no actual precipitation of the dye must take place except in the substance to be stained, and I should be inclined to modify the above theory by omitting stage I, and reading instead of 'coagula produced in stage I' simply 'colloids of fibre-substance.' These bodies usually having a negative charge, it is

1. *Proc. Roy. Soc.* 76 B, p. 225, 1905.
2. *Journ. of Physiology*, 33, p. 359, 1905.
3. *Ber. Deutsche. Chem. Ges.*, 1878, p. 823.
4. *Phil. Mag.*, 38, pp. 206, 218, 1894.
5. *Journ. Chem. Soc.*, 88, p. 1935, 1905.

necessary, in order for them to take up an electro-negative dye, that the latter shall be discharged by the presence of a kation. When the dye is electro-positive, on the other hand, neutral salts are unnecessary and usually deleterious, since both classes of dyes are most sensitive to the kation which would be inhibiting in the case of positive dyes. In certain cases alkali increases the adsorption of these latter, presumably owing to the powerful action of the  $\text{OH}^-$  ion more than counteracting the opposite effect of the kation. The common practice of using basic dyes in strong solution of borax and acid dyes in acid solution is in agreement with this view. It appears, that in the case of certain acid dyes, such as congo-red, the concentration of the electrolyte used in practice is unnecessarily high.

##### 5. *The Staining of Histological Preparations*

Here again the balance of evidence is, as it seems to me, in favour of the adsorption theory.<sup>1</sup> It may be that there are exceptional cases where true chemical combination takes place, but they appear to be rare.

This being so, it is obvious that the part played by electrolytes must be taken into account. If electrolytes are split off from living cells when they die or are injured it is clear why these cells readily take up acid dyes under such conditions ; moreover, since electrolytes are unnecessary when the substance to be stained is electro-negative, it is also clear why living cells can be stained with basic dyes, if, as seems probable, the structures in question have a negative charge. The reaction of the tissues is on the alkaline side of neutrality and, as Hardy has shown, globulin is electro-negative in alkaline solution, and Pauli has shown the same for albumin.

I am not forgetting the work of Overton on the solubility of basic dyes in the 'Plasmahaut,' and the insolubility of acid dyes therein. The action of electrolytes must also, nevertheless, make itself felt.

An interesting case, which at the same time presents considerable difficulty as to its explanation, is the staining of granules in the axis-cylinder of nerve-fibres. These granules are stained by neutral-red as

1. See Alfred Fischer, 'Fixierung, Färbung, etc. d., Protoplasmas.' Jena, 1899.

shown by Macdonald<sup>1</sup>, but only in the position and neighbourhood of an injured spot. They correspond to the distribution of potassium ions, and are interpreted by Macdonald as precipitates of the dye caused by potassium. He finds, in fact, that fairly strong solutions of potassium salts are capable of throwing down the dye. Since neutral-red is a basic dye these results seem at variance with the action of neutral salts on adsorption of these dyes as described in the preceding pages. I thought it of interest therefore to see whether neutral-red behaves differently to the basic dyes on which my experiments were chiefly performed. I found it, however, to behave quite in the same way. I did not, indeed, notice any tendency to precipitation even by 2·6 per cent. KCl. There are evidently, then, some other factors at work in the case of the staining by neutral-red of the granules produced by injury in the axis-cylinder of nerve-fibre.

Since congo-red showed itself to be particularly sensitive to electrolytes it would seem a suitable reagent for the detection of electrolytes, if split off from injured tissue. I have tested the behaviour of nerve-fibres to this dye. It is easily seen, under the microscope, that only the cut ends of the nerve are stained and not the uninjured parts of the fibre. I could not detect any appearance of granules, but did not look for them under a high power. It was to be noted also that connective-tissue fibres took the stain, as I imagine because they were non-living.

The fixation of adsorbed dye by heat has already been pointed out. This fact is of interest in connection with Altmann's method of using acid-fuchsin. The fixation of dye by electrolytes has also been mentioned.

Recently Emil Mayer<sup>2</sup> has shown that the affinity of the Nissl bodies of nerve-cells for basic dyes is abolished by previous treatment with neutral salts. This is in complete concordance with the results I have described.

It occurred to me that, considering the opposite action of neutral salts on electro-negative and electro-positive dyes it should be possible

1. *Proc. Roy. Soc.*, 76, page 325, 1905.

2. *Hofmeister's Beiträge*, 7, p. 560, 1906.

to vary the colour of paper stained in a mixture of two dyes of opposite character by the addition of electrolytes. This proved not to be so, the reason being the formation of dye-compounds, which appeared to be unaffected by electrolytes. One of these dye-compounds, that of eosin and methylene-blue, is well-known to histologists and is generally regarded as a salt of the methylene-blue base with the eosin acid. Similar compounds are formed by most pairs of acid and basic dyes. They are very insoluble in water though soluble in excess of either component. This latter fact suggests the possibility that they are not true compounds but adsorption-compounds of oppositely-charged colloids. As shown by Biltz mutual precipitation occurs in such cases and solution in excess of either colloid. On this account I have devoted some attention to these bodies. In the first place, if true chemical compounds are formed by a process of double decomposition the inorganic components should be found combined together in the solution. I tested this in the case of anilin-blue and methylene-blue by taking about 3 grams of the latter and the molecular equivalent of the anilin-blue. The precipitate was filtered off and the filtrate evaporated to dryness in order to estimate the chloride present. Unfortunately, by an omission to make a note of the amount of the filtrate from the silver chloride, the exact value was not arrived at. From the quantity of silver nitrate required to completely precipitate the chloride, it was obvious, notwithstanding, that practically the whole of the chlorine of the methylene-blue was contained in the filtrate. The precipitate was, therefore, a compound of the methylene-blue base with the anilin-blue acid, the sodium of the anilin-blue having at the same time combined with the chlorine of the methylene-blue. I should hesitate to state definitely whether this kind of combination was inconsistent with a process of adsorption, especially in the light of van Bemmelen's results ; on the face of it, at the same time, it appears more like a true chemical compound. Dr. J. H. Scott informs me that he has noticed that a new absorption-spectrum makes its appearance when solutions of eosin and methylene-blue are mixed. I have repeated this observation in the following way : A solution of methylene-blue was taken of such a strength as

to show distinctly the two dark bands on the red side of the D-line in a certain cell. An equivalent molecular solution of eosin was taken, so that when mixed in equal volume there should be two molecules of methylene-blue to one of eosin. This eosin solution was placed in a cell of equal depth to that containing the methylene-blue and the two cells then placed before the slit of the spectroscope. The combined spectrum of the two dyes was seen. Equal volumes of the two solutions were then mixed, a portion of the mixture at once placed in both cells and these then observed. By taking a double layer it would seem that there would be no change in the spectrum unless chemical changes had taken place. What one sees is that the band of methylene-blue next the D-line has almost completely disappeared, leaving a faint shade. No change is to be seen in the band in the red, nor in the eosin band at F. The same disappearance of the one band of methylene blue may be observed when any other acid-dye such as anilin-blue or congo-red is taken instead of eosin. On the other hand, if thionin or toluidin-blue is taken instead of methylene-blue, no obvious change is produced in the spectrum until precipitation occurs, when, of course the bands become much fainter. I believe the explanation of the disappearance of the methylene-blue band is to be found in the fact that it is the first to disappear on mere dilution. Now when an oppositely charged colloid is added to a solution of the blue the precipitate does not fall at once and it might be supposed there was no important change ; but, if the experiment is made in a vessel through which a beam of bright light is passing, the track of the beam is barely visible in the methylene blue, but almost immediately on adding the other dye the beam begins to appear, gradually becoming brighter as the particles increase in size. In point of fact then, methylene blue is really taken out of solution, and the first band to disappear would be the one next the D-line. The other thiazin-dyes, so far as investigated, do not show the same behaviour on dilution, the various bands all disappear or fade in nearly the same proportion.

These dye-compounds, although almost insoluble in water, are soluble in alcohol, so that it might be supposed that by taking a strong

alcoholic solution of a methylene-blue compound, one should see both the methylene blue bands. This is not so, and is due to the fact that in alcoholic solution of methylene-blue the band next the D-line is not to be seen at all distinctly. If the solution showing only the other band is gradually increased in depth or concentration, it is seen that this band broadens out until it reaches the D-line, but there is no indication of a gap between the bands. A fact which confirms the interpretation I have given of the change in the methylene-blue spectrum produced by combination with an acid-dye, is that scharlach R. in weak alcoholic solution produces the same change, and also a precipitate of a dye-compound. Now scharlach R. is an indifferent dye, not forming salts, but forms an electro-negative colloidal solution ; so that it would seem that here, at all events, the precipitate must be an adsorption compound. A similar state of affairs is to be observed in the case of rosolic acid. Here the possibility of double decomposition is also absent, except, of course, that by mass-action a small proportion of methylene blue rosolate might be formed. The only instance I have found where there is a real change in the spectrum is in the mixture of methylene-blue and picric acid ; picric acid is, however, a powerful reagent, and much weight cannot be laid on this fact as far as concerns the question before us. In mixing methyl-violet and an acid-dye no change is to be observed in the absorption spectrum.

The absence of change in the absorption-spectrum of these dye-compounds does not, of course, exclude the possibility of their being true chemical compounds.

With regard to the properties of these bodies, they are indiffusible, as such, through parchment-paper. In dilute watery solution they appear to be dissociated ; for, on dialyzing the eosin-methylene-blue compound, for example, it will be seen that after a time eosin begins to pass through, and is ultimately followed by the methylene-blue. Since this observation was made on a solution of the pure body, which had been thoroughly washed with cold water, the eosin and methylene-blue must have passed out as the free acid and base respectively. This dissociation is also shown by the fact that, at  $100^{\circ}$ , the solution is a fairly good conductor of the electrical current, and is electrolyzed by a constant current.

Although these compounds are, no doubt, colloids, they do not appear to carry a charge—or only a very small one. Electrolytes have a very slight effect in the sense of retarding adsorption. In the boundary apparatus the behaviour of anilin-blue-methylene-blue under electric stress is difficult to interpret, but since the phenomena are the same in neutral acid, or alkaline solution, it does not seem to be a question of electric charge. What happens is this : after the current has passed for some time it will be seen that electrolysis has occurred, so that on the anode side there is a layer of anilin-blue solution, and on the cathode side one of methylene-blue. The upper boundaries of these two are at the same level in both limbs, but the methylene-blue layer being deeper than the anilin-blue it makes the level of the unaltered compound on the cathode side lower than on the anode side, so that it appears as if negatively charged and moving to the anode. The behaviour to electrolytes, if any at all, is, on the contrary, in the sense of a positive charge ; so that I am inclined to think that the behaviour in the electric field is, in some way, due to different velocities of the two ions. In the case of the eosin-methylene-blue there was no similar difference in level of the unaltered dye, so that it may be that here the two ions are more nearly equal in velocity.

This absence of proof of any definite electric charge makes the compounds less interesting from the theoretical point of view in some respects. But it is, I suppose, what might be expected if these bodies are formed by the mutual neutralization of electro-positive and electro-negative colloids.

On the whole it seems impossible to give, in the present state of knowledge, a decided answer to the question as to the nature of these dye-compounds.

#### 6. *Antitoxins*

It has been shown by Craw<sup>1</sup> that the combination between toxin and antitoxin follows more closely in its nature that of adsorption than that of chemical combination. The puzzling fact known as the 'Danysx-von-Dungern phenomenon' is, for example, satisfactorily

1. *Journ. of Hygiene*, 5, p. 115, 1905, and *Proc. Roy. Soc.*, 76 B, p. 179, 1905.

explained by what I have called in the earlier part of this paper the 'law of adsorption.' In illustration I may cite one experiment:

Two filter-papers of equal size (12 cm. diam.) were each cut into eight pieces. Two flasks, each containing 50 c.c. of a dilute congo-red solution were taken, and to one of these the whole of one paper was added at once, to the other piece by piece at intervals of about twelve hours. After all the paper had been added the amounts taken up from the solution were:

Added altogether	...	...	...	37 %
„ piece by piece at intervals	...	...	48 %	

It is advisable in this experiment to wait a considerable time between the addition of each piece of paper on account of the slow attainment of equilibrium in the case of congo-red and paper.

#### 7. *The part played in enzyme-action*

The interest of adsorption phenomena in this region is in connection with the combination between enzyme and substrate, which so much recent work indicates as an indispensable condition of attack. The fact that enzymes are carried down when a colloidal precipitate is produced in a solution containing them, has been long known and used as a means of preparation. But whether the union between a more or less specific enzyme and the body hydrolyzed under its influence is to be looked upon rather as a true chemical combination is a matter of dispute.

Certain experiments have been made by Dauwe<sup>1</sup> as to the taking up of enzymes by colloids. These were done chiefly on pepsin and various proteins, a few on the taking up of pepsin by agar and of emulsin by coagulated egg-white. Dauwe comes to the conclusion that the process is not one of adsorption, since the same weight of boiled egg-white takes up the same amount of pepsin whether the egg-white is in large pieces or fine powder. He considers that the hypothesis of solid solution applies to the case better. It seems to me that this conclusion is scarcely justified. Coagulated egg-white is not a homogeneous solid body, but porous, like charcoal, and therefore its active surface must not

1. Hofmeister's *Beiträge*, VI. p. 426, 1905.

be restricted to the external surface of the pieces of various size. Porous solids like charcoal, even in lumps, adsorb gases, and that not only on their surfaces but throughout their substance. Dauwe, indeed, appears to have thought of this possibility, but regards adsorption as the same thing as solid solution in such cases.<sup>1</sup> The distinction is, surely, analogous to that between true solution and colloidal solution. Although recent investigations teach that we must not draw a hard and fast line between these two kinds of solution, there is no doubt that the molecules or particles in suspension change their properties as their dimensions increase beyond what are ordinarily called molecular, and that they begin to have the properties, due to surface, of matter in mass. In the same way, as I think, we ought to keep the name 'solid solution' for such cases as alloys of metals, and give the name adsorption not only to the taking up of substances from their solutions by surfaces, in the usual sense of the word, but also by such surfaces as those forming the walls of pores. The criterion by which any given case is to be decided, is, of course, the way in which the relative amounts taken up vary as the concentration of the body taken up changes.

Dauwe rejects the hypothesis of chemical combination on the ground that the enzyme taken up can be extracted again by an appropriate solvent, for instance, pepsin from coagulated egg-white by a solution of egg-white. He regards the process as one of solid solution, or relative solubilities of the enzyme in the substrate and water, and brings forward in support of this view the experiments of Reichel and Spiro<sup>2</sup> on the apparent loss of rennet in the process of clotting of milk. These observers showed that the disappearance of the enzyme was to be accounted for by the taking up of it by the clot. Now, as I understand their results, they are rather in favour of adsorption. If it were a case of solid solution the percentage loss would be the same whatever the rennet concentration, but if one looks at the table on page 481 of the second paper referred to, it is seen that the percentage loss steadily *increases* as the concentration of the

1. *Loc. cit.*, p. 443.

2. Hofmeister's *Beiträge*, VI, p. 68, 1905, and VII, p. 479, 1905.

enzyme falls ; in other words the more dilute the solution the more adsorption takes place. This is particularly well shown by the last experiment on the table. The mathematical expression given is also in accordance with this interpretation.

If the union of enzyme and substrate follows the law of adsorption it would be expected that there would be found some effect of this law on the relation of activity of enzyme to its concentration. In my paper on *The Kinetics of Tryptic Action*,<sup>1</sup> I showed that the form of the function in question varied according to the stage of the reaction, but if we take the early stages in which the action is most rapid there seems to be some effect suggestive of an adsorption process. The initial linear stage is very short, so that we may omit it, and, if we compare the relative times taken by concentrations of trypsin varying as 8, 4, 2, and 1 to produce an increase of conductivity of 1800 gemmhos in caseinogen solution, we find the values are :

Relative trypsin content		Time taken in minutes
8	...	54
4	...	79
2	..	126
1	...	233

so that the lower concentrations of trypsin are relatively more active. It is somewhat remarkable that the value of the exponent found in another experiment was 1.42 to 1.67<sup>2</sup> and this is the same as that given by Reichel and Spiro in their paper for the 'Teilungsfactor' of rennet, viz., 1.5 to 1.67. Whether this is merely an accidental coincidence I am not prepared as yet to state, the subject being still under investigation.

I have myself made one or two experiments similar to those of Dauwe, which are perhaps of sufficient interest to give here. It was shown by W. A. Osborne that calcium caseinogenate does not pass through a porous clay filter, trypsin, on the contrary, does do so. If then, we mix solutions of trypsin and calcium caseinogenate, the

1. *Archives des Sciences Biologiques*. Tome XI, Suppl. p. 261. St. Petersburg, 1904.  
2. Page 26 of the reprint.

'compound' formed should prevent the appearance of trypsin in the filtrate. This I find to be the case. It may be said that what we have here is an instance of mutual action of colloids, but, as I think I have been able to show in the previous pages, there is no essential difference between this action and adsorption by solids from watery solution. On the other hand, it might be held by some, that the case is one of true chemical combination. To test this view I performed a similar experiment, taking caseinogen and malt amylase, and found that this enzyme is also held back by caseinogen, so that it appears to be merely adsorbed. This interpretation of the union of enzyme and substrate does not, of course, exclude a certain degree of specific relation between a particular enzyme and the substrate hydrolyzed by it, as we have seen there is considerable evidence of specific adsorption. It is probable, moreover, that it does not hold, in the same degree, for such enzymes as invertase, maltase, or lactase, when the substrate hydrolyzed is not colloidal, and where there seems to be a very close relationship between the chemical structure of the enzymes and the bodies split by them.

In another way the results described in the present paper are of interest in connection with enzymes. I refer to the action of electrolytes. From the work of Cole,<sup>1</sup> McGuigan,<sup>2</sup> and others, it follows that there is a certain opposition between the action of kations and anions. This indicates that enzymes are possibly electrically charged colloids. Victor Henri,<sup>3</sup> in fact, speaks of trypsin as a negative colloid. I have tested the behaviour of a solution of Grübler's trypsin in the boundary apparatus, and found that it does indeed move to the anode, but how far this negative charge is due to the enzyme itself it is naturally impossible to decide until we have in our hands a pure preparation of the enzyme.

It is possible, however, to attack the problem in another way. Caseinogen in solution in alkali is electro-negative, if trypsin is also negative neutral salts, and especially those of bivalent kations, such

1. *Journal of Physiology*, XXX, pp. 202 and 281, 1904.

2. *American Journal of Physiology*, X, p. 444, 1904.

3. *C. R. Soc. de Biologie*, LIX, p. 132, 1905.

as calcium, should increase the adsorption. In some preliminary experiments I have made there seems to be some action of this kind, but it is difficult to obtain the correct relative concentrations so as to avoid precipitation. An experiment was also made on adsorption of trypsin by paper as follows :—

Four crystallizing dishes were taken, each containing a circle of filter paper, in two of them there was a watery solution of trypsin, in the other two a solution of trypsin of the same strength in  $\frac{m}{350}$   $\text{CaSO}_4$ . After standing in a cool place for 24 hours, a paper from the watery solution was taken out, drained for 1 minute, placed in a flask, and heated to  $100^\circ$  in a steam sterilizer ; the same was done with a paper from the  $\text{CaSO}_4$  solution. 160 c.c. of 5 per cent. caseinogen in ammonia were added to each, and they were then placed in the thermostat at  $39^\circ$  ; when warmed to the first was added a drained paper from the  $\text{CaSO}_4$  trypsin, and to the second a similar one from the watery solution. The electrical conductivities were determined at intervals. There was not a very great difference between the two, but what there was showed that rather more trypsin had been taken up by paper under the action of  $\text{CaSO}_4$ . The times taken to reach a change of 800 gemmhos were 165 minutes for the trypsin from  $\text{CaSO}_4$  solution, and 192 minutes for that from watery solution. A similar experiment taking a  $\frac{m}{350}$  solution of toluidini-blue instead of  $\text{CaSO}_4$ , was unsuccessful on account of mutual precipitation of the dye and trypsin. The precipitate, when filtered off and washed, showed itself to have considerable tryptic power. This subject requires further investigation.

The importance of the study of adsorption and surface-action in general in connection with the enzyme-action is emphasized by Bredig<sup>1</sup> in his article on 'Chemical Kinetics' in the *Ergebnisse* of Asher and Spiro.

#### 8. *Oligo-dynamic action*

It has been pointed out by Pauli<sup>2</sup> that the puzzling phenomena called 'oligo-dynamic action' by v. Naegeli, and consisting in toxic

1. *Ergebnisse d. Physiologie*, I, p. 211, 1902.

2. Hofmeister's *Beiträge*, VI, p. 257 (footnote), 1905.

actions of distilled water which has been in contact with polished metal, are, in all probability, due to the presence of colloidal metallic hydroxides. Various solids, such as paper, are known to remove this toxic property. It occurred to me, therefore, that perhaps the adsorption of congo-red which occurs to some extent, even from distilled water, might be due to these hydroxides. I found that the ordinary laboratory distilled water caused considerably more adsorption of congo-red than that which I had prepared myself, using a tin condenser. From this latter, nevertheless, 22 per cent. was taken up. By allowing filter-paper to soak in this water previously the value was reduced to 18 per cent. It did not seem possible to get below this—no doubt the electrolytes dissolved from the glass and the traces remaining in the paper were sufficient to account for the effect.

### VIII. SUMMARY OF RESULTS

1. The hyperbolic form of the curve of adsorption is confirmed.
2. The curve of electrical conductivity of successive distilled water extracts of gelatin has the same form.
3. It is impossible to wash out all the electrolytes from gelatin except by a practically infinite number of changes of water, each change removing a less percentage than the previous one.
4. The electrolytes are, therefore, neither chemically combined nor merely admixed, but in the intermediate form known as adsorption.
5. When gelatin has been washed nearly free from electrolytes it is capable of diminishing the conductivity of solutions of electrolytes in which it is placed. This it does by adsorbing them in a non-ionized condition.
6. The rate at which congo-red is taken up by paper is greatly accelerated by rise of temperature, but the total amount taken up when equilibrium is attained is less the higher the temperature.
7. The temperature-coefficient of the reaction-velocity is extremely low, so that the theory of Nernst as to the part taken by diffusion in heterogeneous reactions seems to apply to the case of adsorption.

8. At low temperatures equilibrium is attained very slowly, at room temperature at least 24 hours being required.
9. The adsorption-compound of gelatin and inorganic electrolytes is also dissociated as the temperature rises.
10. Raising the temperature rapidly to 100° tends to fix congo-red in paper so that it is afterwards extracted by water with considerable slowness.
11. No evidence was obtained of any production of heat in adsorption.
12. The reaction between congo-red and cellulose is reversible, as is also that between gelatin and electrolytes.
13. Dyes forming colloidal solutions are extremely sensitive to electrolytes in regard to their adsorption. The effect seems to be proportional to the degree of their colloidal nature or size of colloid particles.
14. The action of electrolytes may be expressed as follows : In the case of electro-negative dyes, like congo-red, kations facilitate adsorption, anions depress it. In the case of electro-positive dyes like toluidin-blue kations depress, anions facilitate. But in both cases the effect of anions is very small compared to that of kations.
15. The effect of bivalent kations is considerably greater than twice that of univalent kations.
16. Salts of the heavy metals which form positively charged colloidal hydroxides in solution have a very powerful effect in promoting adsorption of electro-negative dyes.
17. There is evidence of the carrying-down with the adsorbed dye of the facilitating ion.
18. The presence of a stable colloid, like gelatin, protects congo-red from the action of electrolytes. A negative charge seems necessary for this action, since egg-albumin, although exercising a similar action in alkaline solution, had the opposite effect in acid solution.
19. The explanation of the action of electrolytes, as well as that of other electrically-charged colloids is to be found in the negative charge of non-conductors, like paper, when immersed in water. (Quincke).

20. The different behaviour of silk and paper towards electro-negative and electro-positive dyes, as also the influence of alcohol on the process, is to be explained by the results of Coehn on the influence of the respective dielectric constants on the charge.

21. When gelatin is precipitated by tannin its adsorbed electrolytes are split off.

22. There is no evidence of a sudden separation of electrolytes at the moment of death. There is a gradual one when a living tissue is warmed from  $11^{\circ}$  C. to  $56^{\circ}$  C.

23. In the process of clotting of blood there is a *diminution* of electrical conductivity, so that ions (probably  $Ca^{++}$ ) disappear from solution.

24. The adsorption affinity of gelatin for acid-fuchsin is greater than that for congo-red, while that of paper is the same for both.

25. A theory of dyeing is suggested on the basis of adsorption in relation to electrically-charged colloids.

26. An explanation, apart from different permeability of the cell, is suggested for the staining of living cells by basic dyes and the non-staining by acid dyes.

27. The nature of the compounds between acid and basic dyes is investigated. They appear to be uncharged colloids, but the evidence as to whether they are colloidal adsorption compounds or true chemical compounds is not decisive.

28. Evidence is brought forward to show that the union between enzyme and (colloidal) substrate is of the nature of adsorption. The adsorption of trypsin by paper is facilitated by calcium sulphate.

TABLE OF DYES

Name	Chemical nature	Molecular weight	Diffusion	Size of Particles	Electric migration	Electrical conductivity in gemmhos	State of solution	Action of					
								H	K	Ca	OH	Cl	SO <sub>4</sub>
Picric acid	Colour acid	229·2	Rapid	Partially sub-microscopic	...	...	?	...	o	...	...	...	...
Rosolic acid	Colour acid	304·2	Very slow	Marked Tyndall phenomenon	To anode in weak alcohol	...	Negative colloid in weak alcohol	...	+	+	...	...	...
Methyl orange	Colour acid	327·37	Fairly rapid	...	Ppt. on anode	...	?	...	...	...	...	...	...
Eosin	K salt of colour acid	647·9	Rapid	A-microscopic	Ppt. on anode	$\left(\frac{m}{100}\right)$	Negative colloid	+	...	+	...	...	...
Acid fuchsin	Na salt (acid) of colour acid	447·44	Slow	...	To anode	...	Negative colloid	...	+	...	...	...	...
Congo red	Na salt of colour acid	696·68	Very slow	Sub-microscopic	To anode	$\left(\frac{m}{100}\right)$	Negative colloid	+	+	+	-	-	No ppt.
Anilin blue	Na salt of colour acid	734·34	Very slow	Sub-microscopic	To anode	$\left(\frac{m}{100}\right)$	Negative colloid	...	+	+	...	...	...
Nigroin	Na salt of colour acid	About 722	Very slow	Sub-microscopic	...	...	...	...	+	+	...	...	...
Scharlach R	'Indifferent' dye	380·36	Nil into weak alcohol	Sub-microscopic	To anode in weak alcohol	...	Negative colloid	...	+	Ppt.	...	...	...
Methylene blue	Chloride of colour base	319·81	Slow	A-microscopic	To cathode	$\left(\frac{m}{100}\right)$	Positive colloid	-	-	+	...	(+)	Ppt. No effect

TABLE OF DYES—Continued

Name	Chemical nature	Molecular weight	Diffusion	Size of particles	Electric migration	Electrical conductivity in gennhos	Kations						Anions				Action of negative colloid positive colloid stable colloid
							H	K	C <sub>a</sub>	OH	Cl	SO <sub>4</sub>	H <sup>+</sup>	(light)	...	...	
Toluidin blue	Chloride of colour base	291·75	Very slow	A-microscopic	To cathode	...	Positive colloid	—	—	—	+	...	...	...	...	...	
Fuchsin	Chloride of colour base	337·8	Slow	Partially sub-microscopic	Ppt. on cathode	...	?	...	o	...	...	...	...	...	...	...	
Fuchsin in anilin water containing free base	Do.	...	Very slow	Sub-microscopic	To cathode	...	Positive colloid	...	—	—	...	...	...	...	...	...	
Methyl violet	Chloride of colour base	(393·85)	Slow	Partially sub-microscopic	...	...	Positive colloid (V. Henri)	...	...	...	...	...	...	...	...	...	
Neutral red	Chloride of colour base	287·82	Very slow	Partially sub-microscopic	...	...	...	—	...	...	...	...	...	...	...	...	
Magdala red	Chloride of colour base	4597·79	Very slow	...	...	...	Positive colloid (V. Henri)	...	—	..	+	...	...	...	...	...	
Safranin	Chloride of colour base	436·78	Very slow	...	To cathode	...	Positive colloid	...	o	o	...	...	...	...	...	...	
Rosin Methylen blue	Compound of + and — dyes	1138·32	Nil as such	...	...	$\left(\frac{m}{1000} \text{ at } 100^\circ\right)$	Colloid uncharged	...	o	o	...	...	...	...	...	...	
Anilin blue Methylene blue	Compound of + and — dyes	995·65	Nil as such	Tyndall phenomenon in weak alc. sol.	o	...	Colloid uncharged	...	...	...	...	...	...	...	...	...	

## NOTES TO TABLE OF DYES

The *molecular weight* of methyl-violet is given as that of the penta-methyl derivative.

*Diffusion.* The words 'very slow' mean that a certain amount had passed through parchment paper by the end of 24 hours.

The statements as to the ultra-microscopic behaviour of dyes in the column headed 'Size of particles' are taken from the work of Michaelis referred to in the text.

'Sub-microscopic' means that the solutions are resolvable into particles.

'A-microscopic' means that they are optically homogeneous.

*Electric migration.* It was difficult to be certain in the case of methylene blue since bleaching occurred by the electrolytic chlorine.

The measurements of *electrical conductivity* were taken at 39° C unless otherwise mentioned.

They are not to be looked upon as absolutely correct, since the dyes were not specially purified, except the eosin methylene-blue compound.

*State of solution.* It is impossible to make definite statements as to certain dyes, e.g., eosin, since they have some properties of colloids, but not all.

The columns headed 'Kations' and 'Anions' refer to the action of these bodies on adsorption by paper. It is to be understood that in all cases the action of Anions is very much less than that of Kations.

The columns 'Negative' and 'Positive colloid' refer to actual precipitation, not adsorption.

The column 'Stable colloid' refers to the effect of this on the facilitation or inhibition of adsorption by kations.

## A COLOUR REACTION OF FORMALDEHYDE WITH PROTEIDS AND ITS RELATION TO THE ADAMKIEWICZ REACTION

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*(Received March 7th, 1906)*

More than a year ago I observed that the addition of a trace of formaldehyde to acetic acid, which had been deprived by distillation<sup>1</sup> of its power to give, with sulphuric acid, Adamkiewicz's<sup>2</sup> reaction for proteids, restored to it its chromogenic power in a very marked degree. As would be expected, it was found that the acetic acid was not essential, the formaldehyde, proteid and sulphuric acid giving by themselves an identical reaction. On increasing the quantity of formaldehyde the reaction failed after a certain percentage of it had been reached. The explanation of this apparent anomaly was found in the presence of small quantities of oxidising substances (nitrous acid, ferric salts) in the ordinary sulphuric acid employed. Pure sulphuric acid and pure formaldehyde<sup>3</sup> produce no colour reaction with proteids, but after the addition of small amounts of oxidising substances to the acid the reaction is obtained, provided that the amount of formaldehyde is very small. When the amount is sufficiently increased, its strong reducing power towards oxidising agents is exerted and prevents the occurrence of the reaction.

Three possibilities present themselves in regard to the action of formaldehyde on proteids in the presence of oxidising agents: (1) The proteid is oxidised first, and its oxidation-product interacts with formaldehyde to produce the colour reaction. This possibility was excluded by

1. F. G. Hopkins and S. W. Cole, *Proc. Royal Soc.*, 68, p. 23, 1901.

2. Adamkiewicz, *Pflüg. Archiv.*, IX, p. 156, 1874; *Ber. d. d. Chem. Ges.*, VIII, p. 161, 1875.

3. Two samples of formaldehyde were met with which gave the reaction with pure sulphuric acid.

This was no doubt due to their containing a trace of hydrogen peroxide (cf. A. Bach, *Moniteur Scient.* [4] XI, p. 479, 1897) or some other active impurity.

negative experiments with proteids treated preliminarily with oxidising agents ; (2) The formaldehyde is oxidised first, giving rise to an intermediate oxidation-product which reacts with the proteid. Decisive evidence for the fact that the reaction can take place in this manner was given by the investigation of the behaviour of the intermediate oxidation-product of formaldehyde. This must be regarded as an aldehydic-peroxide (Diformaldehyde-peroxide-hydrate  $\text{OH}.\text{CH}_2.\text{O}.\text{O}.\text{CH}_2.\text{OH}$ ). This substance is not readily obtainable in a pure state<sup>1</sup>, but its ammonium compound has been prepared and described by A. Baeyer and V. Villiger<sup>2</sup>. It can easily be obtained absolutely pure as a white crystalline powder by the interaction of hydrogen peroxide and formaldehyde in ammonium sulphate solution. An aqueous suspension of this substance was found to react with proteids and pure sulphuric acid, producing the same characteristic colour ring as is obtained in the Adamkiewicz reaction ; (3) Under the usual conditions of the reaction the formaldehyde may combine with the proteid first, the resulting aldehyde-proteid compound (F. Blum,<sup>3</sup> A. Benedicenti,<sup>4</sup> L. Schwarz<sup>5</sup>) being subsequently oxidised. These two stages of the reaction, taking place concurrently under (2), can be demonstrated by the preparation of the pure, colourless aldehyde-proteid compound<sup>6</sup> and its subsequent treatment with oxidising sulphuric acid. The characteristic colour reaction is obtained.

The following oxidising agents have been found to give the reaction in question in the presence of sulphuric acid and formaldehyde : hydrogen peroxide, potassium persulphate, sodium peroxide, platinic chloride, ferric salts, potassium nitrite. The amount of oxidising agent necessary is very small. Nearly every sample of

1. This substance was first found among the products formed by the slow oxidation of ether (L. Legler, *Ber. d. d. Chem. Ges.*, XIV, 1881, p. 602 ; XVIII, 1885, p. 3343.) It is extremely likely that it is also formed in ether exposed to light (Legler, *loc. cit.*), and it seems therefore not improbable that Liebermann's reaction (*Centralbl. d. med. Wiss.*, 1887, p. 321) for proteids after their treatment with active ether is due to the presence in the latter of this substance and not of glyoxylic acid, as supposed by S. W. Cole (*Journ. Phys.*, XXX, 1904, 311).

2. *Ber. d. d. Chem. Gesell.*, XXXIII, 1900, p. 2479.

3. *Zeit. f. Phys. Chem.* XXII, 1896, p. 127.

4. *Arch. f. Anat. u. Physiol.*, 1897, p. 219.

5. *Zeit. f. Phys. Chem.* XXI, 1900, p. 460.

6. The casein-aldehyde compound, washed completely free from formaldehyde, was the one made.

If the aldehyde compounds are prepared according to Schwarz (*loc. cit.*) by means of alcohol and precipitation, it is here specially important to use ether free from peroxide and aldehyde, as otherwise these might give rise to Liebermann's reaction (see supra).

'coml.' sulphuric acid examined was found to give the reaction without needing the addition of an oxidising agent. The amount of formaldehyde necessary is also very small, 1 : 1,000,000 still giving the reaction.

For practical purposes a dilution 1 : 2500 (*i.e.*, 10 c.c. of the commercial 40 per cent. formaldehyde solution to 1 litre) has been generally used. It is preferable to use pure sulphuric acid, and add to it a small quantity of ferric chloride (5 mg. to 100 c.c.), or potassium nitrite (2 mg. to 100 c.c.), or of potassium persulphate, etc. A purple ring soon develops at the surface of contact.

The contact ring of the formaldehyde reaction is absolutely characteristic, and identical in appearance with that of the Adamkiewicz reaction. The colour varies, exactly as in the Adamkiewicz reaction according to the relative amounts of proteids and formaldehyde used from violet to purple. The absorption spectrum of the formaldehyde colouration is identical with that of the Adamkiewicz reaction, showing the same band between D and E.<sup>1</sup>

The reaction with formaldehyde is a general one for proteids and depends, just as has been shown conclusively for the Adamkiewicz reaction by Hopkins and Cole,<sup>2</sup> on the presence of the tryptophane indole group in the proteid molecule. All substances which give Adamkiewicz or Hopkin and Cole's glyoxylic acid reaction also show the formaldehyde reaction. The following proteids have been examined with positive results: crystallised egg-albumin, crystallised serum-albumin, lact-albumin, caseinogen,<sup>3</sup> casein (Hammarsten), fibrin, Witte's peptone, hetero-, proto- and deutero-albumoses, elastin, chondrin, vitellin, keratin, mucin, myosin. Two vegetable proteids, crystallised edestin (from hempseed) and gliadin (gluten), were examined and also gave the reaction, as did commercial preparations of the following enzymes: pepsin, trypsin, diastase, takadiastase. The

1. In preparing the solutions for spectroscopic observation it was found advisable to use a more dilute sulphuric acid than usual in order to prevent the formation of secondary products due to the effect of strong acid upon the proteid (*cf.* also Hopkins and Cole, *loc. cit.*).

2. *Journ. of Physiol.*, XXVII, 1901, p. 418.

3. It is here of interest to note that a test for formaldehyde in milk, introduced by O. Hehner, depends on the formation of a blue ring on the addition of sulphuric acid to formalised milk.

reaction is well shown by crystallised tryptophane (indole-amino-propionic acid) prepared by the method of Hopkins and Cole (*loc. cit.*).

No reaction is obtained with gelatin (absence of indole group). With indole and skatole a rose-red colour, unmistakably different from that obtained with proteids is produced.<sup>1</sup> With tyrosin, only a negative result has been obtained. Under the conditions of the reaction above described the green colour reaction of Denigès<sup>2</sup> is not observed.

The power to produce this colour reaction is limited to formaldehyde, the lowest member of the series. It is given as well by all those compounds which give rise to formaldehyde by the action of acids, such as para-formaldehyde, methylal, hexamethylenetetramine, etc. All the other aliphatic aldehydes examined — acetic, propionic, amylic, oenanthicaldehyde—as well as chloral-hydrate—fail to produce the reaction. That aromatic aldehydes<sup>3</sup> give rise to varying colour reactions with proteids under the influence of strong acids has been shown, by C. Reichl,<sup>4</sup> S. W. Cole,<sup>5</sup> and E. Rohde.<sup>6</sup> These reactions are, however, although depending on the indole group of the proteids (cf. Cole, Rohde, *loc. cit.*), evidently of quite a different character to that of the one with formaldehyde.

#### THE RELATION OF THE FORMALDEHYDE REACTION TO THE ADAMKIEWICZ REACTION

The resemblance in colour, character and absorption spectrum of the formaldehyde reaction to the original Adamkiewicz reaction is striking and unmistakable. When we remember that formaldehyde is formed in considerable quantities by the oxidation of acetic acid

1. The investigation of the behaviour with formaldehyde of indole and its derivatives, as well as of other simple heterocyclic compounds of physiological interest is being continued. The results obtained will be communicated in a later paper, and will, it is hoped, furnish a clue to the real nature of the colouring matter produced in the Adamkiewicz reaction.

2. *Compt. Rend.*, CXXX, 1900, p. 583.

3. It is of interest that the reaction with aromatic aldehydes is obtained with *pure* sulphuric acid, a fact observed by me before Rohde's publication. I had found too that the addition of oxidising agents (impure  $H_2SO_4$ ,  $H_2O_2$ ,  $KNO_3$ , etc.) has a marked influence on the reaction. That potassium nitrite exerts this influence has been stated by F. A. Steensma (*Zeit. f. phys. Chem.*, XLVII, 1906, p. 24) since this paper was written.

4. *Monatshefte f. Chem.*, X, 1889, 317, XI, 1890, 155.

5. *Loc. cit.*

6. *Zeit. f. Phys. Chem.*, XLIV, 1905, 161.

with hydrogen peroxide (Hopkins and Cole, *loc. cit.*), and that hydrogen peroxide is always present in acetic acid exposed to the action of light (Bach, *loc. cit.*), it seems an unavoidable conclusion that formaldehyde is a common impurity of acetic acid. This and the hydrogen peroxide as oxidising agent furnish the necessary conditions for the formaldehyde reaction, which must therefore be regarded as at any rate playing a part in the Adamkiewicz reaction. This conclusion is strengthened by the observation I have often had occasion to make that an acetic acid, which with pure sulphuric acid only gives a relatively slight Adamkiewicz reaction, shows a much more marked chromogenic power with sulphuric acid containing oxidising agents. It is interesting to note that an analogous observation was made as early as in 1888 by E. Salkowski,<sup>1</sup> who, however, offered no explanation for it. He found that the reaction is enormously increased by the addition of a 'minimal' amount of potassium nitrite, but that the opposite effect is produced by large amounts. Both my own and Salkowski's observations are readily explained when one remembers the importance in the formaldehyde reaction of a proper relation between the amounts of formaldehyde and oxidising substance. They show further that in regard to the Adamkiewicz reaction we have to consider not only impurities of the acetic acid but also those of the sulphuric acid.

It is a noteworthy fact that the power to produce the reaction is only exercised by the lowest member of the aliphatic aldehyde series. Hopkins and Cole, as is well known, consider that the lowest member of the aliphatic aldehyde-acid series—glyoxylic acid—is the only constituent in active acetic acid efficacious for the production of the Adamkiewicz reaction. Although from a theoretical standpoint it is extremely difficult to understand why the mere introduction of a carboxylic group should confer chromogenic properties on formaldehyde, the experimental evidence above brought forward does not exclude the possibility that glyoxylic acid, as well as formaldehyde, plays a part in the Adamkiewicz reaction. Once, however, the efficacy of formaldehyde has been established, the question inevitably arises

1. *Zeit. f. Phys. Chem.*, XII, 1888, 221.

whether glyoxylic acid acts as such or only as an intermediate product in the formation of the really active substance, formaldehyde. Hopkins and Cole hold that *pure* glyoxylic acid reacts with proteids in the presence of *pure* sulphuric acid. In spite, therefore, of the ease with which glyoxylic acid gives rise to formaldehyde<sup>1</sup> in the presence of oxidising agents, their reaction could, in the light of my results, in no way depend on the agency of formaldehyde. The methods employed by Hopkins and Cole, however, for the preparation of glyoxylic acid, do not absolutely exclude the presence and activity of oxidising agents. Even the calcium glyoxylate made use of in one case, may from the method of its preparation (from alcohol by means of nitric acid) have been contaminated with the per-acid salt. This is all the more likely if we remember the affinity of lime for hydrogen peroxide.<sup>2</sup> The preparation of their 'reduced oxalic acid' solution by means of sodium amalgam might seem to exclude the possibility of the presence of an oxidising agent, but if we consider what a small quantity of this is necessary and the way in which hydrogen peroxide is formed under the most unexpected conditions, it seems by no means impossible that even here an oxidising agent may have been present.<sup>3</sup>

A method of preparing glyoxylic acid which will exclude the presence and formation of an oxidising agent would seem to be required to settle the question, such a method for instance as the action of water on di-bromo-acetic acid or on di-chloro-acetic ethylester.

I may here mention that I have had three samples from different sources of 'pure' syrupy glyoxylic acid, none of which in freshly prepared solution showed the slightest reaction with proteids and pure sulphuric acid. Although the substances may not

1. Hopkins and Cole obtained from 1 litre of oxidised acetic acid 'considerable' quantities of the osazone of formaldehyde, whilst they only obtained 4 decigrammes of the osazone of glyoxylic acid from 3 litres of the same acetic acid. In all the cases where they obtained the Adamkiewicz reaction with the products of oxidation by means of hydrogen peroxide of various aliphatic acids and aldehydes, and drew the conclusion, without isolating glyoxylic acid, that it had been formed, it is most probable that formic aldehyde had also been produced and took part in the reaction. The substances oxidised by them with hydrogen peroxide were : tartaric, glycollic, glyceric, pyruvic, and paralactic acid, glycerin, glycollic aldehyde, and dextrose.

2. Cf. M. Traube, *Ber. d. d. Chem. Ges.* XXVI, 1893, p. 1471.

3. Hoppe-Seyler (*Zeit. f. Phys. Chem.* II, 1878, p. 22), showed that palladium-hydrogen in presence of water gives rise to hydrogen peroxide. Schönbein noticed long ago that lead amalgam acting in dilute  $H_2SO_4$  produces hydrogen peroxide. M. Traube (*loc. cit.*) has demonstrated that nearly the theoretical yield of hydrogen peroxide is obtained by the action of zinc-amalgam on water.

have been *pure* glyoxylic acid, it was demonstrated that they contained it by various tests (reduction of silver and Fehling's solution, preparation of phenylhydrazin compound). The presence of some deleterious impurity may, of course, be assumed, but the solutions of the acids in question on keeping (exposed to light) became strongly active with pure sulphuric acid and proteids. A sample of glyoxylic acid prepared electrolytically from oxalic acid in sulphuric acid solution gave the reaction in question most markedly, but it is of importance here to remember that very powerful oxidising agents (persulphuric acid and hydrogen peroxide) are known to be produced in the electrolysis of sulphuric acid.<sup>1</sup> It is true that Hopkins and Cole declare that the presence of a trace of hydrogen peroxide suffices to prevent their glyoxylic acid reaction. But my own experience is not in accord with theirs on this point. It was found that if a sufficient amount of glyoxylic acid be present quite a large quantity of hydrogen peroxide can be added without preventing the reaction which does, however, cease when the hydrogen peroxide is in sufficient excess. We find here again the same relationship between the amounts of oxidising agent and aldehyde necessary for the occurrence of the reaction, which exists when formaldehyde itself is employed.

In conclusion it may be pointed out that not only is the formaldehyde reaction an absolutely reliable one, but that it possesses the further advantage of being carried out with a substance now readily procurable in commerce. The reaction has been used for more than a year in the practical classes of this laboratory in place of the somewhat uncertain Adamkiewicz reaction.

#### SUMMARY .

1. Formaldehyde gives rise to a characteristic colour reaction with proteids in the presence of sulphuric acid containing oxidising agents.
2. The same reaction is produced by the interaction of di-formaldehyde-peroxide-hydrate with proteids and pure sulphuric acid.
3. This reaction is due to the formation of a proteid-formaldehyde compound and its subsequent oxidation.
4. The reaction depends on the presence of the Tryptophane (Indole) group in the proteid-molecule.

1. Berthelot, Traube, Baeyer, and Villiger (*Ber. d. d. Chem. Ges.*, XXXIV, 1901, p. 859).

5. The colour obtained is identical spectroscopically with that produced in the Adamkiewicz reaction and in Hopkins and Coles' glyoxylic acid reaction.

6. The Adamkiewicz reaction is not only due to impurities of the acetic acid used, but is influenced by the presence of oxidising agents in the sulphuric acid (nitrous acid, ferric salts, etc.)

7. The question is discussed how far the Adamkiewicz reaction is due to the presence or formation of formaldehyde in the acetic acid used.

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## GLYCOCOLL AND TOTAL MONO-AMINO-ACIDS IN PATHOLOGICAL URINES

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(Received March 28th, 1906)

The present investigation was undertaken with the object of ascertaining whether there were any abnormal changes in the urinary amino-nitrogen of gouty patients. Since its commencement in 1904, papers upon the same subject have been published by Ignatowski<sup>1</sup>, and Embden and Reese<sup>2</sup>, and Plaut and Reese<sup>3</sup>, and Abderhalden and Barker,<sup>4</sup> and F. Erben<sup>5</sup>, and Neuberg and Manasse<sup>6</sup>. Ignatowski<sup>1</sup> found amino-acids to be present in appreciable quantities in the urines of patients suffering from gout, pneumonia, and leuchaemia, while in normal urines only minute traces were observed. Embden and Reese, on the other hand, record the presence of considerable quantities of amino-acids in normal urine. Plaut and Reese fed animals and men on *α*-alanin, and Schittenhelm and Katzenstein<sup>11</sup> on *i*-alanin, and recovered from the urine a large percentage of the amount administered, in the form of *L*-alanin-naphthalin-sulpho-acid. Lipstein<sup>7</sup> has since demonstrated similar amounts of amino-acids in gouty urines to those obtained by Embden and Reese from normal urines.

My present communication concerns itself with the appearance of glycocoll in the urine of children as well as of healthy adults, and with the amino-acid excretion in individuals under varying conditions, rather than with a number of isolated cases. It tends, to some extent, to confirm the findings of Embden and Reese with regard to normal urine. It records the variations in the amount of glycocoll excreted by gouty individuals, but does not satisfactorily clear up the question as to whether the amino-acids are excreted in the free state—thus escaping in small quantities through the renal filter—or whether they appear in the urine in combination with other substances, their ‘pairing’ properties being used for the elimination of bodies otherwise difficult

to excrete. Such questions, as well as those relative to the interesting synthesis and decomposition of amino-acids during the process of metabolism are left for discussion in later papers.

### METHOD

The introduction by Fischer and Bergell of the reaction of amino-acids with  $\beta$ -naphthalin sulphochloride opened the way for a much more exact method of estimating urinary amino-acids than was possible under the earlier era.

Abderhalden and Bergell<sup>4</sup> elaborated a quantitative method for the application of the reaction to urine, and Ignatowski modified the process so that it could be used in hospital laboratories. In their papers, the exact details of procedure and the properties of the several amino-naphthalin compounds are fully detailed, so that it is here needless to repeat them. Embden and Reese further altered the method, in that they more effectually removed the hippuric acid with 20 per cent. acetic ether, and then increased the alkalinity of the fluid in which the final precipitation of the amino-compounds takes place. This latter precaution, they claim, accounts for the increase of the final amino-products in their estimations.

There are one or two points upon which I must remark in this connection. First the urine must be treated in the fresh condition. The following extreme example, culled from a number of determinations, illustrates this necessity.

3,000 c.c. of normal urine were collected, and divided into two portions of 1,500 c.c.:

1,500 c.c. treated immediately	yielded 0,1840 gm. amino-naphthalin acids.
1,500 c.c. " three months later	" 0,0536 " "

A second sample of 3,000 c.c. of abnormal urine was obtained, and divided into similar portions:

1,500 c.c. treated immediately	yielded 0,6073 gms. amino-naphthalin acids.
1,500 c.c. " three months later	" 0,0510 " "

The smaller figures appear to correspond to the possible errors of the method, as ascertained by Abderhalden and Schittenhelm<sup>6</sup> with regard to Frey's work upon glycocoll in cartilage.

Next, the alkalinity during the final precipitation is at present difficult to estimate. In my earlier estimations, I unintentionally used too much alkali, and obtained higher results than those of Ignatowski. Embden later made the solution still more alkaline, and found still larger amounts of amino-compounds. Accuracy is certainly endangered by excessive alkalinity, which permits the formation of naphthalin amides, which have to be removed at a later stage. Abderhalden and Schittenhelm distinctly consider that the use of too much alkali leads to the formation of large quantities of naphthalin-sulpho-amides, which are not too easily removed. Embden and Reese, on the other hand, deem it of little consequence, since the amides can be separated from the naphthalin-sulpho-amides at a later stage. My own experience leads me to regard the excessive alkalinity of the solution as a doubtful procedure, since the separation of the amides introduces complications which are not easy to obviate with accuracy, and introduces a factor—not hitherto allowed for—which may occasion the splitting off of glycocoll, etc., from substances with which it is intimately combined. At best, the present methods, and therefore the results, cannot be considered as more than roughly quantitative.

The method I have adopted is that used by Ignatowski and others, together with these precautions.<sup>10</sup>

The total daily urine was passed into a vessel containing a saturated solution of lead acetate. After complete precipitation, the lead acetate was removed by  $H_2S$ , the latter displaced and the urine concentrated by warming (under  $45^\circ C.$ ) in a vacuum. The filtered solution was then shaken with 20 per cent. acetic ether for three hours, and thrice again with an excess of ether for three three-hour periods. The ether having been separated, the resultant fluid was made just more than slightly alkaline, naphthalin-sulpho-chloride added, and then the mixture was shaken for 12—24 hours, the alkalinity being maintained by frequent testing, and naphthalin-sulpho-chloride being added every three hours. After filtration, concentrated hydrochloric acid was added, and the precipitate obtained was shaken out with ether, and the ether washed with a small quantity of water. The ethereal solution was evaporated, either in a vacuum or by gentle heat, and the residue

dried and weighed before or after being crystallised from hot 20 per cent. alcohol. Or, after strong alkali-sation, the amino-products were separated from the naphthalin-sulpho-amides by solution in dilute ammonia. The purified product was treated in the specified manner with barium chloride, the barium-naphthalin-sulpho-glycin separated and removed, the barium separated by the addition of hydrochloric acid, the resultant naphthalin-sulpho-glycin dried and weighed, and after crystallisation, the melting points of the crystals determined. The melting points varied from  $151^{\circ}$  to  $155^{\circ}$  C. In some instances, the esters were determined. In the glycocoll estimations, there was always a trace of amino-acids after the removal of the glycocoll, but with the quantities of the urine dealt with, it was too small to be treated other than qualitatively.

When shaking out the amino-acids with ether, at the junction of the ether and the mixture containing the acids, there was a mucoid-like brown scum which resisted solution.

#### GLYCOCOLL IN PATHOLOGICAL URINES

The following estimations have been made upon the urines obtained from children who were at the time in-patients of the Manchester Children's Hospital, and I am indebted to Dr. H. Ashby and Dr. H. R. Hutton for permission to utilise their cases, and to Dr. H. Rayner for the collection of the urines. The figures show variations from 0.05 to 0.25 gm. glycocoll during 24 hours.

#### CHILDREN

	Age	Disease	Glycocoll (24 hours)
J. B.	6 years	Mitral disease	0.0050
T. C.	7 "	Splenic anaemia	0.2510
J. W.	7 "	Anaemia (?)	0.0720
E. G.	8 "	Tuberculosis	0.0120
W. L.	8 $\frac{1}{2}$ "	Pleurisy	0.0625
J. S.	10 "	Mitral disease	0.0600
R. H.	10 "	Chorea	0.0135
S. B.	10 "	Mitral disease	0.0060
F. F.	11 "	Persistent vomiting	0.0739
A. W.	12 "	Mitral disease	no trace
M. D.	13 "	" "	"
J. B.	13 "	" "	0.0865

In cardiac disease the quantities are infinitesimal and, in all probability, due to the possible errors of the method.<sup>9</sup> Only in splenic anaemia is there any distinct elimination, and this is in a line with prior observations upon adults. In the cases of anaemia, pleurisy, and vomiting, however, there is certainly some excretion of glycocoll.

## ADULTS

		Weight	Glycocoll per 24 hours
J. W.	Healthy	12 stones	0.0182 gm.
S. R.	Leuchaemia	9 "	0.1280 "
P. P.	Rh. arthritis	9 "	0.1168 "

Here again in leuchaemia and rheumatoid arthritis there is a marked excretion of glycocoll when compared with the amount excreted by a healthy person.

## TOTAL NAPHTHOLIN-SULPHO-AMINO-ACIDS IN URINE

The following estimations show the limits and average of the excretion of the total monoamino-acids in the cases investigated.

	Date	Disease	Naphthalein-sulpho-amino acid gm. per 24 hours
S. Mc.G.	1904	Gout	0.1022—0.5735
J. W.	"	"	0.2440—0.3598
J. W.	1905	"	0.0644—0.3350
Köln (1)	"	"	0.1050 in 500 c.c. of urine ; say 0.2500 in 24 hours
Köln (2)	"	"	0.1800 in 50 c.c. of urine ; say 0.3600 in 24 hours
J. F. S.	"	Diabetes	0.3500
W. W.	"	Normal	0.2785
W. W.	1906	"	0.1365

There is a certain similarity between all the quantities, but the gouty individuals exhibit a somewhat wide range of variation. The amount excreted in diabetes is more than a normal output, and this tends to confirm the recently published work of L. Mohr.

CASES OF GOUT DURING ACUTE ATTACKS AND DURING INTERVALS,  
AND THE EFFECTS OF DIET, ETC.

*T. W.*, male, *aet* 42. Long-standing case with pronounced gouty symptoms. Large tophi on all joints; frequent discharges from tophi on phalanges. Amputation of one leg above knee for secondary suppuration in tophaceous joint.

Date	Naphthalin-sulpho-amino-acids per 24 hours	Interval between acute attack	
		Acute attack	Milk diet
1904. Jan. 19	0.2810 gm.		
" " 22	0.2815 "	"	"
" " 23	0.3598 "	"	"
1905. Dec. 4	0.1024 "	Acute attack	Milk diet
" " 5	0.1400 "	"	"
" " 6	0.2234 "	"	"
" " 7	0.1000 "	"	"
" " 8	0.3350 "	"	"
" " 9	0.0644 "	"	"

In this case an average excretion of amino-acids in the urine was maintained throughout long periods. During the acute attack, the amount was slightly less than that previously ascertained, but the daily elimination was irregular.

*S. Mc.G.*, male. Chronic gout, numerous tophi, few acute attacks.

Date	Naphth-sulpho-amino acids for 24 hours	Interval between acute attacks	
		Milk diet	Meat diet
1904. Oct. 16	0.0870 gm.		
" " 20	0.0628 "	"	"
" " 22	0.1850 "	"	"
" " 26	0.1022 "	"	"
" Nov. 18	0.1840 "	"	"
" Oct. 15	0.4139 "	"	Meat diet
" " 21	0.2630 "	"	Milk diet plus 50 gms. casein
" " 23	0.5735 "	"	Milk diet plus 75 gms. casein
" Nov. 15	1.0073 "	"	Milk diet plus 2 gms. glycocoll

In this instance, the addition of an excess of myo-albumins and of casein was associated with an increased amount of mono-amino acids in the urine, while the administration of glycocoll, which under ordinary conditions is almost entirely oxidised to urea, was followed by a marked increase in the output. The above figures are taken as averages from one experiment extending over 38 days. On several occasions the acids were estimated by Fischer's ester methods, the amounts varying from 0.0200 to 0.0700 gm. glycocoll ester.

The results of these determinations are almost all within the boundaries set forth by Embden and Reese as normal variations. The fallacies of the methods at present available may be responsible for the different findings, but there seems to be little doubt that small quantities of mono-amino acids are present in the urine, either free or combined with other substances. In the cases of gout here investigated, there appear certain variations in the amounts, and these differences are accentuated by feeding upon casein—which contains a large percentage of leucin and tyrosin—and after the administration of glycocoll. Of course these cases are insufficient for any possible interpretation of results. They are merely recorded as a contribution to the accumulation of facts and to indicate the necessity for continued observations. There is a little temptation to compare the figures obtained in conditions of altered and lowered metabolism, such as gout and leuchaemia, etc., with the appearance of glycocoll and tyrosin in the urine when large quantities of these substances are administered by the mouth, but at present there exists no basis for such comparison in the human subject.

It is a pleasure to thank Professor Dr. Minkowski and Professor Dixon Mann for placing three cases at my disposal, and Professor Lorrain Smith for generous provision of laboratory apparatus and reagents.

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## THE INFLUENCE OF X-RAYS ON THE NITROGENOUS METABOLISM AND ON THE BLOOD IN MYELOGENOUS LEUKAEMIA

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During the past two years the medical literature has contained many articles shewing the value of X-Ray therapy in the disease myelogenous leukaemia.

In November, 1905, Sir James Barr asked me to determine the nitrogenous excretion in such cases, and an investigation was made of the nitrogenous metabolism in a case then admitted.

The methods adopted were those originally introduced by Professor Vaughan Harley for the investigation of metabolic changes, and which I have had repeated opportunities of carrying out in his laboratories.

In brief, the observations were carried out thus:—After several days trial diets, the patient was put on a diet containing 25 grams of nitrogen per diem. Each day the diet was accurately weighed and measured by the nurses at the Royal Infirmary, for which trouble and the care they expended my thanks are due to them. At the commencement of the first day of the observation the patient took one drachm of charcoal. From this time the urine was accurately gathered for each day and the first faeces blackened by charcoal shewed the food taken at the commencement of the observation. Similarly at the end of the last day of the observation another one drachm of charcoal was given and the faeces then blackened shewed the food taken after the period of observation, and so were rejected, the faeces in the interim being weighed and estimated.

This method gives also the motor time of the intestine, and as the bowels in this case were opened twenty-four hours after the

charcoal was given, and each day at the same time it was justifiable to presume that the faeces contained the unused nitrogen of the previous day's food.

The total nitrogen in the faeces and urine was estimated by Kjeldhal's method, and the uric acid by the Folin-Hopkin's method. Blood films were examined each day and a leucocyte count made on alternate days.

The investigation was divided into two parts :—

- (1) To show the average nitrogenous excretion of the patient.
- (2) To show the effect of daily exposures to the X-Rays.

The first series of observations shewed the average intake of nitrogen over a period of five days to be 25.67 grams—the average excretion of nitrogen in the urine was 11.39 grams. The nitrogen in the faeces was not determined during these first observations. The average excretion of uric acid was 4.738 grams per diem, thus being within the normal limits. A normal excretion of uric acid was also recorded in two other cases in the Royal Infirmary—one under the care of Sir James Barr, and another under the care of Dr. Abram. This is interesting to note in connection with the statement found in the Text Books of Medicine that the excretion of uric acid in this disease is always increased.

In the second series of observations, during the period of treatment by X-Rays, the following results were obtained .—

	Nitrogenous Intake	Nitrogen in Urine	Nitrogen in Faeces	Total Nitrogen output
1st day	24.27	14.27	3.6	17.87
X-Ray treatment began on second day.				
2nd ,,	20.24	16.6	2.34	18.94
3rd ,,	21.13	19.18	1.5	20.78
4th ,,	13.28	13.25	1.67	14.92
5th ,,	16.01	16.98	.89	17.87
6th ,,	18.20	15.69	1.32	17.01

During the first series of observations the patient's weight remained constant. During the second series of observations the patient lost 1½ lbs. in weight.

At 10 a.m. on the second day of the second series of observations an exposure to the X-Rays was given, lasting ten minutes ; the day, as far as the estimations were concerned, commencing at 6 a.m., so that for twenty hours of that day the metabolism was carried on under the influence of the exposure. Dr. Thurstan Holland (Radio-grapher to the Royal Infirmary) arranged that the exposures which were to be given on each successive day should be given under exactly the same conditions.

The table shows that the first day the nitrogenous intake exceeded the output by 6.4 grams. On the second (after the first exposure) the balance in this direction was only 1.30 grams, and on the third day it was reduced to 0.45 grams. On the fourth day the patient vomited his breakfast immediately after taking it, and when I saw him he was in bed feeling very ill, so it was considered inadvisable to take him to the X-Ray room, or to disturb him by taking blood films, or by making a leucocyte count. His nitrogen intake on this day (allowing for his vomiting his breakfast) was only 13.28 grams, whereas the output in his urine and faeces amounted to 14.92 grams, and the nitrogen balance had, therefore, gone to the other side, making a deficit of 1.64 grams which, therefore, had to come from his tissue proteids to make up the amount excreted. Similarly, on the fifth day, a deficit of 1.84 grams of nitrogen was noted. On the sixth day an attempt to return to the former condition before the treatment with X-Rays was noted, the nitrogenous intake again exceeding the output by 1.19 grams.

The uric acid excreted on each of the above days was as follows :—

1st day	2nd day	3rd day	4th day	5th day	6th day
.5100 grams	.6682 grams	.5610 grams	.9391 grams	1.022 grams	.9513 grams

showing a definite and well-marked increase ; which is especially well marked on the last three days.

It will thus be seen that the action of the X-Rays was to produce a marked increase in the nitrogenous excretion in the urine, and that on two days of the observation proteids were being utilized from the tissues.

The leucocyte counts on the second and fifth days of the first series of observations were 130,000 per *c.m.m.* and 140,000 per *c.m.m.* respectively.

On the first day of the second series of observations 100,000 per *c.m.m.*<sup>1</sup>

On the day following his illness, viz., the fifth day of the second series, a count of 250,000 per *c.m.m.* was recorded, which surprising result caused me to do three successive counts, each with the same result.

On the last day the count recorded 135,000 per *c.m.m.*

This marked increase in the number of leucocytes occurring as it did with increase of nitrogenous excretion and of uric acid, led me to conclude that the synchronous constitutional disturbance had some relation to these changes.

I found on examining the records of other cases similarly treated in the Royal Infirmary that such an increase in the leucocytes within a few days of the commencement of exposure to X-Rays was quite a common event. Out of seven cases of which I have the full records it is noted on five occasions—all within eight days of the first exposure. Ambertin and Beanjard<sup>2</sup> have reported a rise after each sitting, the maximum rise being at the end of the first day; in two cases recently reported by Bruce,<sup>3</sup> a similar variation was found in one case, in the other no count was recorded until twenty days after the commencement of the exposures.

The effect of X-Ray treatment on the temperature of these cases is difficult to determine owing to the complex variations usually found in the disease, but in some instances I find records of rises in temperature synchronous with the X-Ray exposures as in the case of the patient upon whom these observations were made.

When he first had X-Ray treatment in October, 1904, the evening temperatures for the first six days were

99.2° F., 99.6° F., 100° F., 100.2° F., 100.4° F., 100.6° F.  
respectively.

1. The second series of observations commenced one day after the end of the first.

2. *La Presse Medicale*, Aug. 20, 1904, p. 533.

3. *Lancet*, Jan. 27, 1906, p. 211.

Then the temperature fell nearly to normal and remained so with occasional exacerbations until July, 1905, during which time he had been having X-Rays intermittently. In July, 1905, he had a severe illness with high temperatures reaching at times 103° F. Sir James Barr then diagnosed his condition as one of 'X Ray fever.' Two other patients in the infirmary have had similar illnesses.

Senn,<sup>1</sup> the originator of this treatment states that he had to suspend the exposures owing to patients having symptoms of intoxication accompanied by high temperatures.

Bryant and Crane<sup>2</sup> report that in their case after a few days exposure there was a severe constitutional reaction.

The blood films examined each day showed the following changes observed by Dr. R. J. M. Buchanan, which are here described by his permission :

'The films spread badly after exposure to X Rays, and a marked leucolysis is present. The nuclei of the cells are in many instances fragmented, and in others diffusent. The eosinophile myelocyte shews diffusence of granulation. The cells appear as "phantoms" and the so-called spread cells are increased in number.'

In addition to these changes I observed that before the action of X-Rays the leucocyte granules took in a basic stain—Methylene Blue—particularly well, but with the successive exposures this reaction became less and less marked. Dr. Buchanan kindly examined my films, stained others himself, and confirmed the observation. As the basic stain combines with granules of an acidific character the observation shows that the granules become less and less acid during the treatment, a fact interesting to observe in connection with the notable increase of uric acid in the urine.

The observations, I venture to think, show that the exposure to X-Rays of patients suffering from myelogenous leukaemia produces a disintegration of proteid material in the leucocytes, the products formed giving rise—in some cases at least—to grave constitutional disturbance.

1. *Medical Record*, August 22, 1903.  
2. *Medical Record*, April 9, 1904.

That uric acid is the only body produced it is idle to suppose, but the uric acid increase may be taken as an indication of the amount of disintegration going on. Therefore, when X-Ray therapy is being used in this disease it may be suggested that it is advisable to allow the patient sufficient time between each exposure to excrete the toxic products formed, and as indication of this time daily estimation of the uric acid excreted would be of value, and give the physician a guide as to the safe dose of X-Rays to be used.

Gramegua and Quodrone report that exposure of normal rabbits to the X-Rays produced changes in the leucocytes and an increased (though not regular) excretion of uric acid and phosphates. It is, therefore, possible that patients undergoing X-Ray therapy for any reason have these changes produced, and if so the above conclusions might possibly lead to a more rational use of X-Rays as far as their therapeutic dose is concerned.

I have to thank Sir James Barr for providing me with the apparatus for the above research, and also for his kindness and help during its conduction. I have also to thank Dr. Bradshaw and Dr. Abram for the use of the records of their cases in the infirmary.

## SECRETION BY THE RENAL TUBULES IN THE FROG

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### INTRODUCTION

In spite of the large amount of experimental work which has been performed in order to elucidate the function of the renal tubules, it cannot be said that any experiment has yet shown, beyond all question, either that they can secrete or that they can absorb, although it is clear that they must do one or both of these. Nussbaum's experiments still seem to offer the only crucial method of testing the truth of the Bowman-Heidenhain view.

Halsey has fully confirmed and extended Nussbaum's results. He found in ligatured frogs that urea and indigo-carmine were secreted, whereas dextrose, egg-albumin, peptone, and carmine were not. He confirmed the statements of Mosberg and Marcuse that under the influence of phloridzin dextrose is secreted, and showed that the same was true when diuretin was injected simultaneously with dextrose. He found sodium chloride, sodium phosphate, and sodium sulphate regularly present in the urine after they had been injected. These experiments do not prove conclusively that the tubules can secrete. For a microscopical examination of the kidneys showed in every case, without exception, a greater or less number of glomeruli still in circulation.

Owing to the importance of the subject it seemed necessary to perform further experiments of the same kind, with the object of deciding whether or not results similar to those of Nussbaum and Halsey could be obtained when all the glomeruli were out of circulation.

One of us (A.P.B.) has previously confirmed Nussbaum's statement that complete ligature of the arteries supplying the kidneys cut all the glomeruli permanently out of the circulation. Nussbaum's statement that injections of urea into a fully ligatured frog cause a secretion of urine was not confirmed, at any rate when the injections were begun as late as two days after the ligature. It was also found that cutting off the arterial blood supply of the kidneys caused the epithelium of the tubules to degenerate rapidly, presumably owing to a lack of a sufficient supply of oxygen. The failure of urea to cause a secretion of urine might have been due either to the absence of glomeruli in circulation, or to the degeneration of the epithelium.

The present experiments deal firstly with the injection of urea beginning directly after the ligature; the result of this was negative. Secondly, they deal with the questions whether by keeping ligatured frogs in oxygen the degeneration of the epithelium can be prevented,<sup>1</sup> and a secretion of urine then obtained; both of these points are answered in the affirmative.

#### METHOD OF EXPERIMENT

The method of operation was essentially the same as that used previously. The frogs were always anaesthetised with the A.C.E. mixture throughout the operation. Before the operation was begun, the bladder was emptied with a catheter, and the bladder was seen to be empty during the operation. The kidneys were reached through a ventral incision. All the branches given off from the aorta between its bifurcation and the origin of the coeliaco-mesenteric artery were divided by means of the cautery; the coeliaco-mesenteric artery was exposed in its course from the aorta to the spleen, and all vessels given off from it tailwards were divided. The whole operation lasted about half an hour. Male specimens of *Rana temporaria* alone were used, and in all cases the testes and fat bodies were removed.

1. Miss Cullis, *Journ. of Physiol.* (1906), xxxiv, p. 250, states that Brodie suggested the use of oxygen in November, 1905, and that we have carried his suggestion into effect. We wish to point out that our experiments were begun early in October, 1905. Further, in Starling's *Elements of Human Physiology*, 5th edition (1902), p. 451, at the end of the account of Beddard's results this statement is made:—"It is evident that some means must be devised of repeating these experiments while ensuring an adequate supply of oxygen to the tubular epithelium."

At the end of the operation, the anus was tied and a solution of urea, etc., was injected. All injections were made either into the dorsal lymph sac, or under the skin of the thigh. The frogs were placed with water under a large bell-jar filled either with air or with nearly pure oxygen under atmospheric pressure.

The experiments were carried out during the winter months. The frogs were not fed, and were kept in a very cool place. Throughout the course of an experiment the anus remained ligatured, except when the bladder was being catheterised. At the end of the experiment the frog was pithed and the vascular system was well washed out from the aorta with normal salt solution, and then thoroughly injected with a saturated solution of soluble Prussian Blue. Serial sections of each pair of kidneys were cut out of paraffin.

### RESULTS

Three frogs, after the operation, were injected with urea and kept in air (Experiments 1-3). Each received one or two subsequent injections of urea ; they secreted no urine, and at the end of three days were killed. In their kidneys no glomeruli were injected, and the epithelium of the tubules had undergone degeneration. It would be surprising if epithelium, undergoing such a profound change within three days, were capable of secreting at any period after the ligature. If however, as in Halsey's experiments, the ligature was incomplete, urine could be produced not only by the glomeruli in circulation, but also by some of the tubules. For it has been shown previously that the epithelial degeneration does not take place in those portions of a kidney still supplied with arterial blood.

We conclude, therefore, that the positive results obtained by Nussbaum and Halsey were possible only because their ligature was never complete. Whilst Halsey admits that the ligature in his experiments was incomplete, he adds that the number of glomeruli in circulation was too small to be of physiological importance. Nussbaum did not inject the vascular system of the frogs at the end of his experiments, and there is, consequently, no evidence that his ligature was ever complete when he obtained a secretion of urine.

In all the frogs, twelve in number, which were kept in oxygen after the operation, and in which the ligature was found to be complete, the epithelium of the tubules was microscopically normal. It seems clear, therefore, that the degeneration observed in the kidneys of the frogs kept in air is due to a diminished supply of oxygen to the renal epithelium.

In six of these twelve frogs fluid was found in the bladder after injections of urea, alone or in combination with dextrose, phloridzin or  $\text{Na}_2\text{HPO}_4$  (Experiments 4-9). In the other six no fluid, or at most a few drops was found in the bladder. (Experiments 10-15). In one of these normal salt solution alone had been injected, in another urea and  $\text{Na}_2\text{HPO}_4$ , and in four urea and phloridzin.

Before concluding that the six positive results were due to a secretion of urine by the renal tubules, it is necessary to show that the fluid could have reached the bladder by no other route than the ureters. The other possibilities are the anus, the rectum, and secretion by the walls of the bladder itself. The first is excluded by the continuous ligature of the anus, and the other two would seem to be excluded by our six negative results. But in order to exclude definitely the possibility of secretion by the walls of the bladder, two other experiments were performed. (Experiments 16-17). In these two frogs both ureters, the anus and the rectum above the bladder were ligatured and injections of urea, or urea in combination with  $\text{Na}_2\text{HPO}_4$  or phloridzin were given. In neither case was any fluid found in the bladder. The possibility that fluid in the bladder could have been derived from the rectum is excluded by the following considerations:—In no case, *post mortem*, was the rectum found distended with fluid; the drop or two of fluid in the rectum is brown and contains brown flakes, whilst that in the bladder is clear and almost colourless. Further, in two of the positive results (Experiments 8 and 9) the rectum had been tied above the bladder at the operation. This was done because it was found that injections of  $\text{Na}_2\text{HPO}_4$  and phloridzin sometimes set up slight diarrhoea, so that in passing the catheter into the bladder the eye might become fouled with a few brown flakes which might interfere with determinations of the acidity of the urine.

We conclude, therefore, that the fluid obtained from the bladder was urine secreted by the renal tubules, and that secretion of urine can be obtained in fully ligatured frogs, provided they have been kept in oxygen.

No. of Experiment	Material injected	Quantity of urine in twenty-four hours	Acidity	Urine contained
4	Urea ... ...	$\left\{ \begin{array}{l} a 0.5 \text{ c.c.} \\ b 1.0 \text{ c.c.} \end{array} \right\}$	...	Urea and salts
5	" ... ...	1.0 c.c.	...	" "
6	" ... ...	0.5 c.c.	1.9	" "
7	Urea and dextrose	0.5 c.c.	...	Dextrose, urea and salts
8	" phloridzin	0.5 c.c.	2.0	" "
9	" $\text{Na}_2\text{HPO}_4$	$\left\{ \begin{array}{l} a 0.5 \text{ c.c.} \\ b 0.25 \text{ c.c.} \end{array} \right\}$	$\left\{ \begin{array}{l} a 2.4 \\ b 2.5 \end{array} \right\}$	Urea and salts

The table shews the quantity and the composition of the urine secreted by the six fully ligatured frogs. None of them secreted during the first day after ligature, and this is not surprising considering the severity of the operation. Incompletely ligatured frogs, however, may secrete urine within twenty-four hours of the operation. (Experiments 21 and 22). The greatest quantity of urine secreted in a day by a fully ligatured frog was 1 c.c. Observations on normal frogs of the same size kept under the same conditions showed that they might produce as little as 0.25 c.c., and not more than about 1 c.c. in a day. When, however, the normal frogs had received injections of urea, etc., the quantity of urine was four or more times greater than that produced by ligatured frogs after the same injections. This difference may be amply accounted for by the absence of glomeruli in the one case. For whatever difference of opinion there may be as to how the glomerulus works, a consideration of its structure hardly leaves room for doubt that it is a mechanism which can pass out large quantities of water.

The urine secreted by the ligatured frogs contained, in all cases, urea. This was recognised by the production of urea nitrate crystals. It contained also chlorides and sulphates, which were demonstrated by the ordinary qualitative tests. No quantitative estimations of these constituents could be made in the small quantities of urine available.

Urates and phosphates never could be demonstrated even after the injection of  $Na_2HPO_4$ , and the same was true of similar quantities of urine obtained from normal frogs. The urine, like that of normal frogs, gave a reddish colour with a saturated solution of picric acid and 10 per cent.  $NaOH$  similar to that given by kreatinin in solution. But no attempt was made to demonstrate conclusively the presence of kreatinin.

Five fully ligatured frogs had received injections of phloridzin and urea. In one only (Experiment 8) was a secretion of urine obtained. This urine gave a red colour with a solution of ferric chloride similar to that given by the original phloridzin solution, and reduced Fehling's solution readily. No attempt could be made to identify the reducing substance present, but it was considered to be dextrose for the following reasons:—It is recognised that injections of phloridzin produce glycosuria, and it was found that the urine of neither ligatured nor unligatured frogs ever contained a substance which reduced Fehling's solution unless phloridzin or dextrose had been injected.

This experiment shows that phloridzin can produce glycosuria by acting on the renal tubules. It confirms the conclusions reached by Mosberg, Marcuse, and Halsey as the result of similar experiments. It cannot, however, be held that any of these previous experiments have proved the point. For Halsey admits the presence of glomeruli in circulation. And Mosberg in only one of his experiments injected carmine in order to see that his ligature had been complete, and in that experiment he found glomeruli in circulation. He quotes two experiments with phloridzin performed by Marcuse in which also no injection of the vascular system was made.

Although it has been shown that phloridzin can produce glycosuria by an action on the renal tubules, it does not follow that in normal animals there is no action also upon the glomeruli. The fact that it was found difficult in fully ligatured frogs to obtain a secretion of urine after phloridzin, although secretion occurred readily in unligatured ones (Experiment 20), is open to two interpretations. It would be possible to believe that phloridzin acts upon the glomeruli as well

as on the tubules. It might equally well be that the tubule epithelium of ligatured frogs kept in oxygen, although normal in appearance, is not sufficiently normal in function to react to phloridzin or that the epithelium is not in condition to withstand the deleterious action of phloridzin. These experiments seem to favour the latter supposition. For it was found much more easy to obtain a secretion of urine when urea had been injected alone than when the same dose of urea had phloridzin added to it.

Dextrose together with urea was injected into two frogs. In one the ligature was slightly incomplete (Experiment 22), but in the other it was complete (Experiment 7). Both secreted urine which reduced Fehling's solution readily. The injection of 0.1 gram dextrose into a frog of this size leads to hyperglycaemia. For it was found that when this dose had been injected into a normal frog the urine of the next twenty-four hours contained 0.06 gram and for another day reduced Fehling's solution slightly (Experiment 19). We conclude that in a condition of hyperglycaemia the renal tubules can secrete dextrose.

This conclusion is opposed to the results obtained by Nussbaum, Mosberg, and Halsey. It is only in Halsey's experiments that there is any information about the completeness of the ligature. If, as he believes, dextrose is passed out by the glomeruli alone, and in all his experiments glomeruli were in circulation, it is difficult to understand why he never found dextrose in the urine, except when he had given diuretin at the same time. And the same is true of Nussbaum's and Mosberg's experiments. Although in these there is no microscopical examination of the kidneys to show that glomeruli were in circulation, we infer that such must have been the case, otherwise their frogs kept in air would not have secreted urine at all.

Some observations were made upon the acidity to phenolphthalein of the urine of ligatured and unligatured frogs. A method of estimation suitable to the small quantities of urine available had to be employed; and the following was found to give concordant results. Urine was sucked up to the mark 1 on a Thoma-Zeiss white blood corpuscle haemocytometer. The stem of this instrument below the

bulb is divided into tenths up to 1. This volume of urine was blown into a watch-glass and '05 of the same volume of a weak solution of phenolphthalein was added. With the same pipette measured quantities of  $\frac{N}{100}$  NaOH were added, the mixture was stirred with a fine glass rod after each addition and held over white paper in a good light. The first tinge of pink in the fluid was taken at the end point. The urine of frogs is almost colourless; consequently the end point of the reaction is not obscured by the colour of the fluid. The acidity of the urine is expressed in vols. of  $\frac{N}{100}$  NaOH necessary to make 1 volume of urine just alkaline to phenolphthalein.

The acidity of the urine of normal frogs was found to vary widely. Thus one frog (Experiment 18) passed 0.25 c.c. urine with an acidity of 2.3, whilst in another (Experiment 9) the acidity approximated to that of the blood to phenolphthalein, in that the slightest addition of alkali made the urine alkaline. Between these two extremes intermediate figures were obtained in other cases. The most frequent figures were about and generally less than 1. The acidity was influenced in the usual way by the injection of drugs. Thus in Experiment 18 urea injections reduced the acidity from 0.6 to 0.2, and the injection of  $Na_2HPO_4$  raised it to 1.5.

Four estimations were made of the acidity of the urine of fully ligatured frogs. In Experiment 6 after injections of urea the acidity was 1.9, and in Experiment 8 after injections of phloridzin and urea it was 2.0. In Experiment 9 the urine before the operation was only just acid to phenolphthalein, but after ligature and the injection of  $Na_2HPO_4$  and urea it had an acidity of 2.4 and 2.5 on successive days.

We conclude from these results that the tubules can secrete a fluid which is more acid to phenolphthalein than the blood or than the urine turned out by the glomeruli during a glomerular diuresis. Our results confirm those of Dreser. He showed that by injecting acid fuchsin into normal frogs the glomeruli remained uncoloured, and that the fluid and cells of the tubules were red and therefore acid to this indicator. It must be remembered that the uncoloured condition of the glomeruli might be due, when the secretion of urine was small, just as much to the absence of secretion by the glomeruli as to

the secretion by them of an alkaline fluid. When acid fuchsin was injected into ligatured frogs the red colour was still seen in the tubules. From this Dreser concluded that the difference in reaction between blood and urine was due to the secretion of acid radicles by the tubules and not to the absorption of bases from a glomerular filtrate. The only source of fallacy in Dreser's experiments is the possibility that his ligature was not complete. He did not inject the vascular system at the end of an experiment, and as he used a dorsal method of ligature similar to that used by Adami it is certain—as has been shewn by one of us—that his ligature was incomplete. His control experiments in which he tested his method of ligature by injecting vermillion into the anterior abdominal vein, never showed injected glomeruli. This must mean that his method of injection was unable to show with certainty when glomeruli were in circulation.

Our results, like those of Dreser, are not compatible with the view put forward by Cushny, namely, that the normal difference between the reaction of blood and urine is brought about, not by the addition of  $\text{HPO}_4^{2-}$  anions to a glomerular filtrate, but solely by the absorption from that filtrate of  $\text{Na}^+$  kations in combination with  $\text{OH}^-$  or  $\text{HCO}_3^-$ . These experiments on ligatured frogs do not prove that absorption may not play a part, but only that it is not the sole mechanism at work.

It seems at first sight a little surprising, if secretion is a normal function of the renal tubules, that this epithelium after ligature should not secrete spontaneously but should need a powerful stimulus in the shape of large quantities of urea. Nussbaum, Adami, and Beddard have all found that frogs kept in air after ligature do not secrete spontaneously; and this is true even when the ligature is moderately incomplete. But in these frogs much of the epithelium has visibly degenerated and probably none of it is strictly normal. The case seems different, however, with frogs kept in oxygen. In Experiment 15, normal salt solution alone was injected in the hope of obtaining a spontaneous secretion. The frog apparently had bled more at the operation than was thought at the time. The result was negative. The attempt to obtain a spontaneous secretion was not persisted in as our primary object was to obtain positive results if possible. But even

in oxygenated frogs the epithelium can hardly be functionally normal, although it appears so microscopically. For in Experiment 14, in which no adverse circumstance such as haemorrhage was present, injections of urea and  $\text{Na}_2\text{HPO}_4$  failed to produce any secretion of urine. Nor can it be a matter of indifference to the kidney that it should be cut off from its high pressure blood supply.

The frogs in the other four experiments with negative result had received injections of phloridzin as well as of urea. As in two of these (Experiments 10, 11) not more than the very slight unavoidable haemorrhage had taken place at the operation and there had been no post-operative oozing of blood, we believe that the main adverse factor at work was damage done by phloridzin to an already depressed epithelium.

#### CONCLUSIONS

The experiments which we have carried out seem to prove two facts, namely, (1) that the renal tubules of the frog can secrete; and (2) that they secrete urea, chlorides and sulphates, and dextrose during hyperglycaemia and after injections of phloridzin. It is possible that they secrete kreatinin and, judged by the reaction of the urine, phosphates—in fact most of the normal urinary constituents. This fully confirms the view of the function of the tubules put forward originally by Bowman and then by Heidenhain.

According to the view of Ludwig, elaborated by Starling, the fluid turned out by the glomeruli is a mechanical filtrate and must be isotonic and identical with the blood plasma except for the absence of proteids. This view entails the further supposition that the main function of the tubules is to absorb water, dextrose, bases, etc., and so to concentrate the glomerular filtrate. In order to explain the production, during some diureses, of urine with  $\Delta$  very much less than that of the blood plasma, Starling has suggested that the tubules may then secrete an extremely hypotonic fluid. We have found, however, that, when the tubules do secrete in response to urea and water, they turn out a urine which contains besides other crystalloids certainly as much urea as that of normal frogs. Although this observation does not disprove the view that the glomerulus is a mechanical filter, it

renders filtration extremely unlikely, and, therefore, necessitates a belief that the glomerular epithelium secretes. And again, if both the glomeruli and the tubules can secrete, there is no longer any necessity to believe that the tubules also absorb. But, whilst there certainly is no experiment which proves that the tubules can absorb, there is equally none which shows that they are unable to do so. Absorption by the tubules must be left an open question to be proved or disproved by future experiments. It is clear, however, that no view of the function of the renal tubule can be considered adequate and final which does not explain the function of its different parts corresponding to their variations in structure.

Starting from the observed fact that the tubule can secrete and from the suppositions that the glomerulus also secretes and is a mechanism whose most important function is to pass out any excess of fluid, and remembering that the only known difference between the urines secreted by the glomerulus and tubule, is that one is always and the other never acid to acid fuchsin, we would suggest the following working hypothesis of the secretion of urine :—Both the glomerular and tubule epithelia definitely secrete urine, and in all probability both can secrete much the same urinary constituents in solution. At times when the quantity of fluid to be secreted from the blood is comparatively small the urine represents for practical purposes the secretion of the tubules, because the epithelium covering the glomeruli is insignificant in extent as compared with that of the tubules. When, however, as during saline diureses, large quantities of water and salts have to be passed out, the mechanism which removes rapidly the bulk of this fluid is the glomeruli ; consequently at the height of such a diuresis the urine secreted by the glomeruli very greatly exceeds that being secreted at the same time by the tubules. During saline diuresis the urine approximates more and more to the composition of blood plasma minus proteid, whereby the maximal amount of material is excreted with minimal expenditure of energy by the kidney cells. This power of the glomeruli to get rid of fluid is a true secretion which is generally greatly assisted by increased blood flow through the kidney, but can take place independently of the vascular changes. In

other words the primary factor in such a diuresis is not the vascular change but the state of activity of the glomerular epithelium. If once it is admitted that vascular changes alone cannot set up diuresis by increased glomerular filtration, then it seems necessary to believe with Eckhard that the state of activity of the renal epithelium can be influenced directly by the central nervous system, and that the nerve endings described by Berkeley in the renal epithelium represents the terminations of this nervous supply. Otherwise it seems impossible to explain a diuresis caused by disturbance of the medulla oblongata; for there is no reason to believe that a change in the composition of the blood takes place in such a nervous disorder. It is further probable that all diureses are not solely and entirely of glomerular origin, but that some depend largely upon an increased activity of the tubular epithelium.

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## DETAILS OF THE EXPERIMENTS

## SOLUTIONS USED FOR INJECTION.

- (a) Urea solution=10 per cent. urea in normal salt solution.
- (b) Dextrose and urea solution=5 per cent. dextrose and 10 per cent urea in water.
- (c) Phloridzin and urea solution=5 per cent. phloridzin in the urea solution.
- (d)  $\text{Na}_2\text{HPO}_4$  solution=5 per cent.  $\text{Na}_2\text{HPO}_4$  in water.
- (e) Dextrose solution=10 per cent. dextrose in water.

## EXPERIMENT 1

1905

Oct. 31. Usual operation. Emptied bladder. Injected 1 c.c. urea solution.  
 Placed in air.

Nov. 2. No urine. Injected 2 c.c. urea solution. Tied anus. Air.

," 3. No urine. Washed out and ligatured the vascular system. Microscopically. No injected glomeruli. Epithelium throughout much degenerated.

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### EXPERIMENT 2

1905

Nov. 3. Usual operation. Very little haemorrhage at operation. Emptied bladder. Tied anus. Injected 2 c.c. urea solution. Placed in air.

„ 4. No urine. Tied anus. Injected 2 c.c. urea solution. Air.

„ 5. No urine. Tied anus. Injected 2 c.c. urea solution. Air.

„ 6. 11 a.m. Frog lively. Injected 1 c.c. urea solution. 5 p.m. Pithed frog. *Post-mortem*. Viscera of normal colour and not pale. Two drops of blood-stained urine found in bladder. Washed out and injected the vascular system. Microscopically. No injected glomeruli. Epithelium moderately degenerated throughout.

### EXPERIMENT 3

Nov. 10. Usual operation. Some haemorrhage from one of the renal arteries during the operation. Emptied bladder and tied anus. Injected 1 c.c. urea solution. Placed in air.

„ 11. Injected 1 c.c. urea solution. Air.

„ 12. No urine. Tied anus. Injected 1 c.c. urea solution. Air.

„ 13. No urine. Frog rather feeble. *Post-mortem*, bladder found empty. All viscera very pale. Much blood in the peritoneal cavity. Washed out and injected the vascular system. Microscopically. No injected glomeruli. Epithelium very degenerated throughout.

### EXPERIMENT 4

Nov. 14. Usual operation. Emptied bladder and tied anus. Injected 2 c.c. urea solution. Placed in oxygen.

„ 15. No urine. Tied anus. Injected 2 c.c. urea solution. Oxygen.

„ 16. 0.5 c.c. urine in bladder. Tied anus. Injected 2 c.c. urea solution. Oxygen. Urine contained urea, chlorides, and sulphates.

„ 17. 1.0 c.c. urine, which contained urea, sulphates, and chlorides. Frog fairly lively, but generally oedematous. *Post-mortem*. Viscera not pale. Rectum empty. Washed out and injected vascular system. Microscopically ; no injected glomeruli ; epithelium normal in appearance.

### EXPERIMENT 5

Nov. 14. Usual operation. Very little haemorrhage at operation. Emptied bladder, and tied anus. Injected 1 c.c. urea solution. Placed in oxygen.

„ 15. Two drops of blood-stained urine in bladder. Tied anus. Injected 2 c.c. urea solution. Placed in oxygen.

„ 16. 1.0 c.c. clear urine, containing urea, sulphates, and chlorides. Frog lively. *Post-mortem*. Viscera not pale. Rectum empty. Washed out and injected the vascular system. Microscopically ; no injected glomeruli ; epithelium normal in appearance.

EXPERIMENT 6  
1905

Nov. 17. Usual operation. Some haemorrhage at operation. Emptied bladder, and tied anus. Injected 2 c.c. urea solution. Placed in oxygen.

," 18. No urine. Tied anus. Injected 2 c.c. urea solution. Oxygen.

," 19. No urine. Tied anus. Injected 2 c.c. urea solution. Oxygen.

," 20. 0.5 c.c. clear urine. Frog fairly lively. *Post-mortem*. Viscera pale and much blood-stained fluid found in the peritoneal cavity. Rectum empty. Washed out and injected vascular system. Microscopically ; no injected glomeruli ; epithelium normal in appearance. Urine contained urea, chlorides, and sulphates. Gave a reddish colour with picric acid and caustic soda, similar to that given by solutions containing kreatinin. Phosphates and urates could not be demonstrated. Reaction was about neutral to neutral litmus paper. Acidity to phenolphthalein. 1 vol. urine + 1.9 vol.  $\frac{N}{100}$  NaOH was just alkaline to phenolphthalein.

## EXPERIMENT 7

Nov. 28. Usual operation. Very little haemorrhage at operation. Emptied bladder and tied anus. Injected 2 c.c. urea and dextrose solution. Placed in oxygen.

," 29. Injected 2 c.c. dextrose and urea solution. Oxygen.

," 30. 0.5 c.c. clear urine. Frog appeared moribund. *Post-mortem*. Rectum empty. Viscera were of normal colour. Washed out and injected vascular system ; microscopically ; no glomeruli injected ; epithelium normal in appearance. Urine reduced Fehling's solution readily. It contained urea, chlorides, and sulphates, and was neutral to neutral litmus paper.

## EXPERIMENT 8

Dec. 5. In addition to the usual operation the rectum was ligatured above the bladder. Very little haemorrhage at the operation. Emptied bladder and tied anus. Injected 2 c.c. phloridzin and urea solution. Placed in oxygen.

," 6. Injected 2 c.c. phloridzin and urea solution. Oxygen.

," 7. 0.5 c.c. clear urine. Tied anus. Injected 2 c.c. phloridzin and urea solution. Frog lively but rather oedematous. Placed in oxygen. Urine reduced Fehling's solution readily, and gave a red colour, with a solution of ferric chloride. It contained urea, sulphates, and chlorides. It gave a red colour with picric acid and caustic soda. Phosphates and urates could not be demonstrated. Acidity to phenolphthalein ; 1 vol. urine + 2.0 vols.  $\frac{N}{100}$  NaOH was just alkaline.

## SECRÉTION BY THE RENAL TUBULES IN THE FROG 269

1905

Dec. 8. No urine. Frog moribund. *Post-mortem* viscera not pale. Rectum empty. Frog generally oedematous. Washed out and injected vascular system ; microscopically ; no glomeruli injected ; epithelium normal in appearance.

### EXPERIMENT 9

Dec. 5. In addition to the usual operation the rectum was ligatured above the bladder. Very little haemorrhage at the operation. Emptied bladder and tied anus. Injected 2 c.c. urea solution. Placed in oxygen. Urine drawn off at the time of the operation was alkaline to neutral litmus paper and became alkaline to phenolphthalein on the slightest addition of  $\frac{N}{100}$  NaOH.

," 6. No urine. Tied anus. Injected 1 c.c.  $\text{Na}_4\text{HPO}_4$  solution and 1 c.c. urea solution. Oxygen.

," 7. 0·5 clear urine. Tied anus. Injected 1 c.c.  $\text{Na}_4\text{HPO}_4$  solution and 1 c.c. urea solution. Frog lively but rather oedematous. Placed in oxygen. Urine contained urea, chlorides, and sulphates. Gave a red colour with picric acid and caustic soda. It neither reduced Fehling's solution nor gave a reaction with ferric chloride. Phosphates and urates could not be demonstrated in it. Acidity to phenolphthalein ; 1 vol. urine + 2·4 vols.  $\frac{N}{100}$  NaOH just alkaline.

," 8. 0·25 urine. Frog lively but oedematous. *Post-mortem*. Viscera not pale. Rectum empty. Washed out and injected vascular system. Microscopically ; no glomeruli injected ; epithelium normal. Urine. Acidity to phenolphthalein. 1 vol. urine + 2·5 vols.  $\frac{N}{100}$  NaOH just alkaline.

### EXPERIMENT 10

Nov. 18. Usual operation. Very little haemorrhage at the operation. Emptied bladder and tied anus. Injected 2 c.c. phloridzin and urea solution. Placed in oxygen.

," 19. No urine. Tied anus. Frog lively. Injected 2 c.c. phloridzin and urea solution. Oxygen.

," 20. No urine. Tied anus. Frog lively. Injected 2 c.c. phloridzin and urea solution. Oxygen.

," 21. One drop of clear urine drawn off from bladder. Frog lively and not oedematous. *Post-mortem*. Bladder and rectum empty. Viscera not pale. Washed out and injected vascular system. Microscopically ; no glomeruli injected. Epithelium normal in appearance.

## EXPERIMENT 11

1905

Dec. 1. Usual operation. A little arterial haemorrhage at operation. Emptied bladder and tied anus. Injected 2 c.c. phloridzin and urea solution. Placed in oxygen.

„ 2. Injected 2 c.c. phloridzin and urea solution. Oxygen.

„ 3. Two drops of urine found in bladder. Frog fairly lively. Tied anus. Injected 2 c.c. phloridzin and urea solution. Oxygen.

„ 4. No urine. Frog appeared to be moribund and was slightly oedematous. *Post-mortem.* Bladder and rectum empty. Viscera not pale. Washed out and injected vascular system. Microscopically; no glomeruli injected; epithelium normal in appearance.

## EXPERIMENT 12

Nov. 23. Usual operation. Very little haemorrhage at the operation. Emptied bladder and tied anus. Injected 2 c.c. phloridzin and urea solution. Placed in oxygen.

„ 24. No urine. Tied anus. Injected 2 c.c. urea solution. Oxygen.

„ 25. Three drops of urine in bladder, which gave no reaction with ferric chloride. Tied anus. Injected 2 c.c. phloridzin and urea solution. Oxygen.

Nov. 26. One drop of urine in bladder. *Post-mortem.* Much blood-stained fluid in peritoneal cavity. Viscera pale. Bladder and rectum empty. Washed out and injected vascular system. Microscopically; no glomeruli injected; epithelium appeared normal.

## EXPERIMENT 13.

Nov. 24. Usual operation. Some arterial and venous haemorrhage at the operation. Emptied bladder and tied anus. Injected 2 c.c. phloridzin and urea solution. Placed in oxygen.

„ 26. No urine. Tied anus. Frog lively and oedematous. Injected 2 c.c. phloridzin and urea solution. Oxygen.

„ 27. No urine. Frog very oedematous. *Post-mortem.* Bladder empty. Viscera rather pale. Washed out and injected vascular system. Microscopically; no glomeruli injected; epithelium appeared normal.

## EXPERIMENT 14

Dec. 9. In addition to the usual operation the rectum was ligatured above the bladder. Very little haemorrhage at operation. Emptied bladder and tied anus. Injected 2 c.c. urea solution. Placed in oxygen.

„ 10. Injected 2 c.c. urea solution. Frog fairly lively. Oxygen.

„ 11. No urine. Tied anus. Frog lively. Injected 1 c.c. urea solution and 1 c.c.  $\text{Na}_2\text{HPO}_4$  solution. Oxygen.

## SECRETION BY THE RENAL TUBULES IN THE FROG 271

1905

Dec. 12. No urine. Frog feeble and rather oedematous. *Post-mortem.* Viscera not pale. Bladder and rectum empty. Washed out and injected vascular system. Microscopically; no glomeruli injected; epithelium appeared normal.

### EXPERIMENT 15.

Dec. 1. Usual operation. Slight venous haemorrhage at operation. Emptied bladder and tied anus. Injected 2 c.c. normal salt solution. Placed in oxygen.

„ 2. Injected 2 c.c. normal salt solution. Oxygen.

„ 3. No urine. Tied anus. Frog lively and rather oedematous. Injected 2 c.c. normal salt solution. Oxygen.

„ 4. Five drops of rather blood-stained urine in bladder, which contained urea. Frog very oedematous. *Post-mortem.* Much blood-stained fluid in peritoneal cavity. Viscera pale. Bladder and rectum empty. Microscopically; no glomeruli injected; epithelium normal in appearance.

### EXPERIMENT 16

1906

March 13. Tied both ureters and the rectum above the bladder and tied anus. Injected 2 c.c. urea solution. Placed in air.

„ 14. Injected 2 c.c. urea solution.

„ 15. No urine. *Post-mortem.* Bladder and rectum empty. Ureters and rectum properly ligatured.

### EXPERIMENT 17

March 13. Tied both ureters and the rectum above the bladder. Emptied bladder and tied anus. Injected 2 c.c. urea solution. Placed in air.

March 14. Injected 2 c.c. urea solution.

„ 15. Injected 1 c.c. urea solution and 1 c.c.  $\text{Na}_2\text{HPO}_4$  solution.

„ 16. Injected 2 c.c. phloridzin and urea solution.

„ 17. No urine. Frog lively but rather oedematous. *Post-mortem.* Bladder empty. Rectum above colon contained a few drops of a brown fluid with numerous brown flakes in it. Ureters and rectum properly ligatured.

### EXPERIMENT 18

Feb. 9. Normal frog. Emptied bladder and tied anus. Placed in oxygen throughout the experiment.

„ 10. 0.25 c.c. clear urine. Acidity to phenolphthalein; 1 vol. urine + 2.3 vols.  $\frac{N}{100}$  NaOH just alkaline. Injected 1 c.c.  $\text{Na}_2\text{PO}_4$  solution. Tied anus.

1906

Feb. 11. 2 c.c. clear urine, which contained urea, chlorides, and sulphates. Phosphates and urates could not be demonstrated. It did not reduce Fehling's solution nor give any reaction with ferric chloride. Acidity to phenolphthalein ; 1 vol. urine + 2.9 vols.  $\frac{N}{100}$  NaOH just alkaline. No injection. Tied anus.

,, 12. 1 c.c. clear urine. Acidity to phenolphthalein ; 1 vol. urine + 0.6 vol.  $\frac{N}{100}$  NaOH just alkaline.

,, 14. 4.5 clear urine. This did not reduce Fehling's solution nor give a reaction with ferric chloride. Acidity to phenolphthalein ; 1 vol. urine + 0.2 vol.  $\frac{N}{100}$  NaOH just alkaline. No injection. Tied anus.

,, 15. 2 c.c. urine. Acidity to phenolphthalein ; 1 vol. urine + 0.3 vol.  $\frac{N}{100}$  NaOH just alkaline. Injected 1 c.c.  $\text{Na}_2\text{HPO}_4$  solution. Tied anus.

,, 16. 1.5 urine, which did not reduce Fehling's solution. Acidity to phenolphthalein ; 1 vol. urine + 1.5 vols.  $\frac{N}{100}$  NaOH just alkaline. Tied anus.

,, 18. 0.25 c.c. urine. Acidity to phenolphthalein ; 1 vol. urine + 0.4 vol.  $\frac{N}{100}$  NaOH just alkaline. Injected 1 c.c. urea solution. Tied anus.

,, 19. 0.75 c.c. urine. Acidity to phenolphthalein ; 1 vol. urine + 0.4 vol.  $\frac{N}{100}$  NaOH just alkaline. Injected 1 c.c.  $\text{Na}_2\text{HPO}_4$  solution. Tied anus.

,, 20. 3.5 urine. Acidity to phenolphthalein ; 1 vol. urine + 0.5 vol.  $\frac{N}{100}$  NaOH just alkaline.

## EXPERIMENT 19

Feb. 21. Normal .0.. Emptied bladder and tied anus. Placed in air throughout experiment.

,, 22. 1 c.c. urine which did not reduce Fehling's solution. Injected 1 c.c. dextrose solution. (1 g.). Tied anus.

,, 23. 2.5 urine, which gave no reaction with ferric chloride. The sugar was estimated in this by Pavly's method. 2.3 per cent. dextrose was found, which in 2.5 urine would amount to about 0.06 g. No injection. Tied anus.

,, 24. 2 c.c. urine which gave a slight reduction with Fehling's solution.

## EXPERIMENT 20

Feb. 23. Normal frog. Emptied bladder and tied anus. Placed in air throughout experiment.

## SECRETION BY THE RENAL TUBULES IN THE FROG 273

1906

Feb. 24. 1 c.c. urine, which neither reduced Fehling's solution nor gave a reaction with ferric chloride. Injected 2 c.c. phloridzin and Tied anus. urea solution.

„ 25. 2·5 c.c. urine which was acid to neutral litmus paper, and gave a red colour with ferric chloride. It contained 0·21 per cent. dextrose estimated by Pavý's method. The injections of phloridzin were continued for a week. The urine contained dextrose throughout. On the fifth day severe diarrhoea was set up.

### EXPERIMENT 21

Feb. 7. Usual operation. Very little haemorrhage at operation. Emptied bladder and tied anus. Injected 1 c.c. urea solution. Placed in oxygen.

„ 8. 1 c.c. urine. Frog very sluggish, possibly due to cold weather, possibly to haemorrhage. *Post-mortem*. Much blood in the peritoneal cavity. Viscera pale. Washed out and injected the vascular system. Microscopically; no glomeruli injected except at the extremities of both kidneys where numerous glomeruli contained the injection; epithelium normal.

### EXPERIMENT 22

Feb. 24. Usual operation. Very little haemorrhage at operation. Emptied bladder and tied anus. Injected 1 c.c. dextrose solution and 1 c.c. urea. Placed in oxygen.

„ 25. 0·5 c.c. urine which reduced Fehling's solution strongly. Tied anus. Injected 1 c.c. dextrose solution and 1 c.c. urea solution. Oxygen.

„ 26. Few drops of urine which reduced Fehling's solution. *Post-mortem*. Viscera not pale. Washed out and injected vascular system. Microscopically; no glomeruli injected except at the anterior end of both kidneys; epithelium normal.

## A STUDY OF THE PATHOLOGICAL VARIATIONS IN THE ACIDITY OF THE GASTRIC CONTENTS, ESPECIALLY IN RELATIONSHIP TO MALIGNANT DISEASE

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In a recent paper in the *Proceedings of the Royal Society*<sup>1</sup> we have drawn attention to the frequent absence or reduction in amount of the free hydrochloric acid of the gastric secretion in malignant disease whatever be the situation of the growth, and have referred this diminution of the free acid not to local conditions in the stomach but to altered conditions in the blood plasma.

Since the publication of this paper we have extended our observations from seventeen to thirty-four cases of carcinomata situated elsewhere than in the stomach, and have made determinations of the acidity in twenty other cases of patients not suffering from malignant disease.

We were induced to make the latter series of observations so as to obtain a more nearly analogous control than that of the four healthy laboratory workers given in our previous paper, and also on account of the contradictory results contained in the extensive literature of the subject. The differences obtained by different observers are, no doubt, due in great part to the use of different methods and indicators, to the small number of cases tested by most of the observers, and to the reliance placed on qualitative rather than quantitative results.

The same quantitative methods have been employed in each of our series of cases, under as nearly as possible like conditions of experimentation so as to make them strictly comparable with one another.

1. *Proc. Roy. Soc., B*, Vol. 96, 1905, p. 138.

The methods used have been described at length in the previous paper, and further statement may accordingly be now omitted.

The examination of the twenty non-malignant hospital cases has shown that the acidity of the gastric secretion is very sensitive to alterations in the general health and condition, as is obvious on comparing the average acidity in the case of the four laboratory workers (Table V) with that in the case of the hospital patients (Table IV). But there still remains a striking difference between the malignant and the non-malignant cases, as is seen on comparing Table I with Table IV. Thus, in the cases of carcinoma, the Günzburg Reagent shows an entire absence of free hydrochloric acid in twenty-three out of thirty-four cases, or in 67.6 per cent., and the amount of free hydrochloric acid is only appreciable (above 0.05 per cent.) in four cases (Nos. 23, 28, 29, and 31). On the other hand, although the non-malignant cases show much lower averages than obtained for the laboratory workers, there is complete absence, as shown by the Günzburg test, in only five out of the twenty cases, or in 25 per cent., and the amount lies above 0.05 per cent. in half the cases.<sup>1</sup>

The average amounts of free hydrochloric acid in the non-malignant cases lie between two and three times as high as in the malignant cases for all the methods, excepting the Mörner-Sjöqvist method, which gives them much more closely together.

The ratios of the average amounts of acidity in the two series of cases may be expressed as follows :—

	Malignant	Non-malignant
Total acidity	= 816 : 1,453	= 0.56
Di-methyl indicator	= 352 : 925	= 0.38
Günzburg's reagent	= 163 : 502	= 0.32
Methyl acetate method	= 286 : 630	= 0.45
Mörner-Sjöqvist method	= 637 : 774	= 0.82

For purpose of comparison with the others, we have included in the tables of malignant cases the seventeen cases given in our previous paper, and we have placed in separate tables from the carcinoma cases the few cases of sarcoma and rodent ulcer which we have been able to examine.

1. It is clear from these figures that the presence or absence of free hydrochloric acid cannot be regarded as a diagnostic sign of any great value. All that can be said is that in the majority of malignant cases it is absent, and in the majority of non-malignant cases it is present. The same statement holds for cancer of the stomach wall itself.

TABLE I.—CARCINOMATA

No.	Sex	Age	Situation	Period of digestion in hours	Phenol-phthalicin	METHYL ACETATE METHOD				
						D-methyl	Guinzburg	Mörner-Sjögren	Initial	Final
1	F.	49	C. of uterus	1	0.0219	Negative	Negative	—	—	—
2	M.	..	C. of prostate	1	..	..	Negative	—	—	—
3	F.	71	C. of uterus	1½	0.0036	Negative	Negative	0.0066	—	—
4	F.	50	C. of uterus	1	0.1460	0.0821	0.0368	0.0146	—	—
5	F.	65	C. of liver	1½	0.0018	Negative	Negative	0.0000	—	—
6	F.	32	C. of rectum	1	0.0584	Negative	Negative	0.0013	0.0036	0.95
7	F.	..	C. of tongue	1½	0.0293	0.0186	0.0109	0.0000	0.4	2.2
8	M.	61	E. of floor of mouth	1	0.0018	Negative	Negative	0.0000	0.1	0.0
9	F.	49	Coll. C. of mesentery	1½	0.1058	0.0401	0.0072	0.0738	-0.1350	1.55
10	F.	59	C. of breast	1½	0.0182	0.0091	0.0036	0.0023	0.0000	0.25
11	F.	59	C. of breast	1½	0.0511	0.0219	0.0016	—	0.0000	0.85
12	M.	65	E. of cheek	1	0.0018	Negative	Negative	0.0044	0.0000	0.025
13	F.	66	C. of breast	1	0.0548	0.0019	Negative	0.0020	-0.0402	0.8
14	M.	63	E. of cheek	1	0.2847	Negative	Negative	0.1479	0.2044	2.7
15	M.	57	C. of tongue	2	0.0365	Negative	Negative	0.1140	-0.0657	0.4
16	F.	45	C. of uterus	2	0.0182	Negative	Negative	-0.0620	0.3	0.2
17	F.	77	E. of hand	1	0.1460	Negative	Negative	0.0207	+0.0839	1.8
18	F.	69	E. of cheek	1½	0.0018	Negative	Negative	0.0921	-0.0164	0.05
19	M.	54	E. of lip	1	0.0949	0.0328	Negative	0.0602	-0.0255	1.5
20	F.	43	C. of rectum	1	0.1666	0.0912	0.0446	0.0984	-0.0255	2.6
21	M.	60	E. of jaw	1½	0.0219	0.0182	Negative	0.0438	-0.0182	0.3

TABLE I—Continued.—CARCINOMATA

No.	Sex	Age	Situation	Period of digestion in hours	Phenol-phthalain	Di-methyl	Günzburg	Mörner-Sjöqvist	METHYL ACETATE METHOD				
									Initial	Final	Increase	$\kappa \times 10^5$	Percentage HCl
22	M.	53	E. of Penis	1½	0.1131	0.0657	Negative	0.0827	-0.0584	1.5	5.0	42.832	0.03687
23	M.	59	E. of hand	1	0.2810	0.1898	0.1313	-	-	-	-	-	-
24	F.	63	E. of vulva	1	0.1533	0.1022	Negative	-	-	-	-	-	-
25	F.	67	C. of breast	1½	0.0973	Negative	Negative	-	-	0.15	0.25	0.1	0.801
26	F.	55	C. of breast	1	0.0949	0.0657	0.0355	0.0338	+0.0273	1.2	6.7	5.5	0.00069
27	F.	48	...	1	0.0109	Negative	Negative	-	-	-	-	-	0.04094
28	F.	45	C. of uterus	1	0.2007	0.1351	0.0949	0.1648	0.0000	2.8	15.6	12.8	134.573
29	M.	68	E. of lip	1	0.2239	0.1277	0.085	0.1504	+0.0292	3.2	21.9	18.7	247.490
30	M.	46	E. of face	1	0.0182	0.0073	Negative	Negative	-0.0584	-	-	-	-
31	M.	35	E. of tongue	1	0.2080	0.1642	0.1277	0.1134	-0.0182	2.8	18.5	15.7	182.450
32	F.	38	C. of uterus	1	0.0365	Negative	Negative	-	-	0.5	0.8	0.3	0.11561
33	F.	52	C. of breast	...	0.0073	Negative	Negative	-	-	0.1	0.3	0.2	0.21262
34	M.	45	C. of rectum	1½	0.0730	Negative	Negative	-	-	-	-	-	0.000133
Averages	...	...	...	...	0.0352	0.0163	0.0637	-0.0679	...	...	...	...	0.02865

**NOTE TO TABLES.**—The titration figure to phenol-phthalain gives the total acidity, including acid combined with protein and other organic substances capable of acting as weak bases, the figure to diamethyl gives approximately the total *free* acid, inorganic and organic, but not that in combination; the figure to Günzburg gives the free inorganic acid, chiefly hydrochloric; the Mörner-Sjöqvist method gives the total amount of chloride not combined with fixed bases; the figure after incineration the organic acids previously in combination minus ammonia previously in combination; and the last five columns the concentration of the secretion in hydrogen ions measured by the velocity of hydrolysis of methyl acetate—the first of these columns gives the titration before hydrolysis; the second the titration after hydrolysis for eight hours at 40° C; the increase in the third column shows the amount of acetic acid set free from the methyl acetate by the hydrogen ions of the sample of gastric juice; the fourth column gives the velocity constant in such case; and the fifth column the percentage of hydrochloric acid which would produce an equal effect.

TABLE II.—CASES OF SARCOMA

Sex	Age	Situation	Period of digestion in hours	Phenol-phthalein	Di-methyl	Günzburg	Mörner-Sjögren	METHYL ACETATE METHOD		
								Initial	Final	Increase
F.	43	General in liver, etc. (lympho-sarcoma)	1 1/4	0.0146	Negative	—	—	—	—	—
M.	61	Neck	1	0.1861	0.0036	0.0072	0.1533	—	—	—
M.	35	Thoracic parieties (round celled angio-sarcoma)	1 1/2	0.2263	0.1496	0.0949	0.1441	3.2	15.7	12.5
M.	56	Neck	1	0.1715	0.0474	Negative	—	—	—	—

TABLE III.—CASES OF RODENT ULCER

Sex	Age	Situation	Period of digestion in hours	Phenol-phthalein	Di-methyl	Günzburg	Mörner-Sjögren	METHYL ACETATE METHOD		
								Initial	Final	Increase
M.	69	Face	1	—	—	Negative	—	—	—	—
M.	63	Face	1	0.2774	0.1752	0.0803	—	—	—	—

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TABLE IV.—HOSPITAL CASES—NON-MALIGNANT (ALL MALES).

No.	Age	Disease	Period of digestion in hours	Phenol-phthalein	Di-methyl	Günzburg	Mörner-Sjögren	METHYL ACETATE METHOD					
								Initial	Final	Increase	$K \times 10^5$	Percentage HCl	
1	65	Chronic Bronchitis	1	0.0684	0.0438	0.0292	...	-0.0146	...	...	...	...	
2	65	Chronic Bronchitis	1	0.1418	0.1058	0.0730	0.0965	-0.0403	2.0	11.7	9.7	93.170	
3	35	Apoplexy	1	0.1418	0.0657	0.0971	-0.0365	2.0	11.4	9.4	89.568	0.07695	
4	78	Healthy	1	0.0146	Negative	...	...	0.2	0.3	0.1	0.776	0.00067	
5	44	Old fracture of thigh	1	0.1460	0.0693	Negative	0.0821	-0.0328	2.0	3.5	1.5	11.954	0.01027
6	49	Ulcer of leg	1	0.2680	0.1095	Positive	0.0633	+0.0073	2.7	11.3	8.6	80.255	0.06895
7	45	Injured shoulder	1	0.2519	0.1898	0.1022	0.1353	-0.0109	3.5	18.2	14.7	164.372	0.14152
8	56	Injured scalp	1	0.109	Negative	Negative	-0.0474	0.2	0.0	0.000	0.00000	0.00000	
9	45	Old hip disease	1	0.2628	0.1825	0.1095	0.1064	-0.0474	3.4	13.1	14.7	164.372	0.14152
10	57	Ulcer of leg	1	0.1825	0.0985	0.0401	0.0799	-0.0182	2.65	9.4	6.75	57.884	0.04972
11	41	Ulcer of leg	1	0.1533	0.0803	0.0182	0.0611	-0.0365	2.2	6.1	3.9	31.326	0.02691
12	43	Abscess of neck	1	0.0328	Negative	Negative	...	0.45	1.1	0.65	4.893	0.000420	
13	31	Syphilitic eruption	1	0.1679	...	Positive	...	...	2.3	12.2	9.9	91.783	0.07885
14	49	Sprained arm	1	0.1679	...	Positive	...	...	2.3	14.9	12.6	126.373	0.10857
15	45	Ulcer of leg	1	0.2117	0.1715	0.1350	0.1096	-0.0036	2.9	18.4	15.5	171.703	0.14751
16	38	Tuberculous knee	1	0.2591	0.2080	0.1423	0.1109	0.0000	3.6	18.6	15.0	169.914	0.14590
17	34	Tuberculous thigh	1	0.1204	0.0766	0.0109	0.0144	-0.0474	2.0	6.0	4.0	33.539	0.02880
18	19	Tuberculous knee	1	0.1715	0.1314	0.0766	0.1403	-0.0109	2.5	11.0	8.5	79.199	0.06880
19	47	Tuberculous hip	1	0.0474	Negative	Negative	0.0669	-0.0073	0.6	0.8	0.2	15.55	0.00133
20	23	Tuberculous pelvis	1	0.1460	...	Positive	...	...	2.0	4.5	2.5	20.322	0.01750
Averages	...	...	...	0.1453	0.0935	0.0502	0.0774	-0.0231	...	...	...	0.06354	

TABLE V.—CONTROL CASES IN NORMAL INDIVIDUALS

Sex	Age	Period of digestion in hours	Phenol-phthalein	Di-methyl	Günzburg	Mörner-Sjögren	Initial	Final	Increase	$K \times 10^5$	Percentage of HCl	
M	25	Healthy	1	0.2117	0.1789	0.1579	0.0078	3.05	17.9	14.85	167.300	
M	38	"	1	0.3285	0.2884	0.2571	0.0000	4.8	25.5	20.7	305.740	
M	23	"	1	0.3139	0.2482	0.1862	0.3296	-0.3876	4.0	21.1	17.1	208.248
M	28	"	1	0.2628	0.2336	0.2117	0.2212	-0.0311	3.9	24.7	20.8	309.134
Averages	...	...	...	0.2792	0.2373	0.2007	0.2414	-0.0342	...	...	...	0.21279

In our previous paper, we drew attention to the fact that the Mörner-Sjoqvist method in some cases gave a higher result than the Günzburg or Methyl-acetate methods, and this difference is still more strikingly shown in the longer series of cases. Also, although the figure for the total acidity (to phenol-phthalëin) is very much higher than that given by the di-methyl, Günzburg, and Methyl acetate, indicating the probable presence of organic acids, yet a method specially designed for detecting and estimating such acids, failed to show any, giving instead a negative figure. This method consisted in neutralising to phenol-phthalëin, incinerating and again titrating. In this process, any organic acids would be changed first into salts, and then on incineration into alkaline carbonates ; as a result the ash would have been alkaline, and the back titration would have shown the amount of organic acid plus organic salts in the sample of gastric contents. In the actual result of experiment it was found, however, that the ash was rarely alkaline ; not only so, but on adding *excess* of alkali beyond the neutral point to phenol-phthalëin, there occurred an actual loss of alkali upon incineration.

Both the discrepancies in the Mörner-Sjoqvist method and the failure to show any organic acid might receive a common explanation on the supposition that there is present in such samples of stomach contents salts of inorganic acids with ammonia or organic bases.

For the ammonia or organic base being driven off in the process of incineration, the inorganic acid with which it was previously combined would combine with barium from the barium carbonate in the Mörner-Sjoqvist method, so increasing the amount of soluble barium salt, and hence ultimately the weight of the precipitate of barium sulphate. Also in the incineration with alkali for the purpose of obtaining the organic acid, the inorganic acid would similarly combine with and neutralize alkali and (provided the amount of inorganic acid combined with organic bases or ammonia exceeded the amount of organic acid combined with fixed inorganic bases) would give rise to such negative figures as we have obtained in the great majority of our cases.

This was the explanation which we put forward in our former

paper, but determination in such a sample in which the negative figure was large, of the amount of ammonia by Schlössing's method gave a very small amount, entirely inadequate to explain the negative figure.

The base in combination is therefore not ammonia or a volatile organic base, or it would have been detected in the Schlössing process.

Other substances which might be present in the fluid from the test meal in feeble combination with acid are the proteins and the products of their digestion, albumoses and peptones. Hydrochloric acid in combination with such bodies would not show itself to the Günzburg and di-methyl tests, and only in a very diminished degree in the case of the methyl-acetate hydrolysis method, on account of the much lowered ionization resulting from the combination of the hydrochloric acid with the protein ; while such hydrochloric acid would be given completely by the phenol-phthalein or Mörner-Sjöqvist methods. In the case of the Mörner-Sjöqvist method, on incineration in the presence of excess of barium carbonate the protein is destroyed, and the hydrochloric acid previously in combination with it acts upon the barium carbonate, forming barium chloride, exactly similarly to the free hydrochloric acid, and hence this amount appears as acid in the result. In the case of the phenol-phthalein the hydrogen ion concentration necessary to maintain the protein-hydrochloric acid combination is sufficiently high to affect the indicator and associate it, and hence the pink colour due to the phenol-phthalein ion does not appear until sufficient alkali has been added to entirely break up the protein-hydrochloric acid combination. In contrast with this, in the case of the di-methyl indicator we have a stronger solt to dissociate, and the protein-hydrochloric acid combination is able to exist at a hydrogen ion concentration which still leaves the indicator showing the alkaline colour (yellow), and hence the change from the acid colour (red) at first seen when either free hydrochloric acid or free organic acid is present in the test meal fluid, to the alkaline colour (yellow) occurs when all the free acid has been neutralized, and while the protein-acid combination still exists in solution.

Accordingly, the presence of protein in the test meal fluid would form an explanation of the differences in value given by the different methods. In our opinion, however, this is not the only factor, since the amount of protein as shown by the biuret test is often very small.

Another factor which might produce the divergences seen in the different methods is the presence of acid salts of less dissociated inorganic acids, instead of hydrochloric acid, such, for example, as the primary phosphates of the alkalies ( $MH_2PO_4$ ).<sup>1</sup>

Such salts possess a very low concentration in hydrogen ion ; as a result, their end point, or neutral point, to one indicator is very different to that with another. For example, in titrating phosphoric acid with an alkali, the neutral point with phenol-phthaléin lies at the point corresponding to the presence of  $M_2HPO_4$ , while in titrating with di-methyl it lies at  $MH_2PO_4$ . Hence if the test meal fluid contained any phosphate it would show quite a different acidity with these two indicators.

An experiment which we carried out to test the effect of the presence of phosphates upon the Günzburg reagent gave interesting results, showing that this is also affected by the phosphates, and only begins to react sharply and typically when there is sufficient acid present to form free phosphoric acid.

Ten cubic centimetres of deci-molecular solution of mono-sodium phosphate were taken, and to this deci-normal hydrochloric acid was added, one c.c. at a time, the Günzburg test being carried out after each addition. It was found that the acid phosphate alone gave a completely negative result, and continued to do so with 1, 2, 3, and 4 c.c. of successively added deci-normal hydrochloric acid ; at 5 c.c. there began to appear a faint pink colour which only gradually became more marked as more acid was added, and so with successive tests, until 10 c.c. of deci-normal acid in all had been added, which is the amount necessary to completely set free the phosphoric acid. Beyond this limit, addition of a single c.c. caused a marked increase in the intensity of the pink colour.

1. This explanation was first suggested to us by Mr. R. C. Cowley.

The gradual development of the test as hydrochloric acid was added, in presence of acid phosphate, furnishes a possible explanation of what we had observed in many of the cancer cases when titrating in the opposite direction with decinormal alkali to find out when the free hydrochloric was neutralized. Instead of the sharp reaction which one finds with dilute hydrochloric acid, or in the gastric juice of normal cases, one observes in malignant cases that as more and more alkali is added, there takes place instead of a sharp disappearance of the pink colour a slow fading of it in the successive tests so that it becomes somewhat difficult to identify the exact point at which the free acid has been neutralized.

Similarly in the reaction to phenol-phthalëin of the stomach contents of malignant cases after incineration in the presence of excess of alkali we had found the usual sharp reaction replaced by a very slow fading out of the pink colour of the phenol-phthalëin.

#### DISCUSSION OF RESULTS AND CONCLUSIONS

The chief result obtained in the series of analyses of gastric contents given above is that the percentage of hydrochloric acid is very sensitive to the general condition of the body, and is generally reduced in all cases of illness or enfeeblement.

In addition to this, in malignant disease there is a more pronounced drop in the degree of acidity, so that in the majority of cases free hydrochloric acid is entirely absent, and in practically all cases there is a very marked reduction.

Such absence, or reduction in percentage, of hydrochloric acid is in no wise related, as had been previously supposed, to the presence of a malignant growth in the gastric mucosa leading to a local action and perversion of the acid secreting function. This is shown by the fact that the percentage of absences in our series of carcinomata, in which the growth has been situated in widely different regions of the body, is as high as in most of the series of cases of carcinoma of the stomach recorded by earlier observers.

If we have as the result of these experimental observations to turn away from the view that the reduction or absence of acid is due to a

local effect upon the gastric cells, the only remaining cause is an alteration in the pabulum brought to the acid secreting cells by the circulating nutrient fluid, as a result of which the acid secreting power of these cells is enfeebled or destroyed. Such an action might be brought about either by an organic toxic agent which altered the metabolism of the cell ; or by an alteration in the balance of the inorganic ions of the plasma, and consequently of the lymph, as a result of which the concentration in acid or hydrogen ions diminished, so causing the work on the part of the cell of separating an acid secretion to be increased.

It must not be lost sight of that the latter of these two effects may be involved in the former, because the organic constituents of the plasma, particularly the proteins, are capable of acting either as feeble acids or feeble bases, and accordingly an alteration in the constitution of the protein molecules of the plasma (or of the protein constituents of the cells themselves) whereby these become more strongly acidic or basic in their properties than normal, would give rise to a different ionization in the plasma, whereby its concentration in hydrogen ions would be increased or decreased.

Investigations in the case of the leucocytes by means of acidic and basic stains have shown that the protein colloids which they contain vary in their degree of acidity or basicity, and that these properties change with pathological alterations. It is highly probable that the other cells of the body also can and do undergo similar variations in the degree to which their colloidal protein contents are capable of acting as weak acids or bases, and so altering the degree of acidity or alkalinity of the lymph bathing them. Also the action of the secreting cells of the kidney will depend upon the power of the protein constituents for combining with acid or base, for it is probably only the uncombined inorganic ions of acidic or basic character which are removed, while those combined with or adsorbed by the protein are unaffected, and left in circulation.

In considering the ionization of the plasma and the effects of variations in this upon the action of secreting cells (such as the acid secreting cells of the stomach), it is accordingly difficult or impossible

to entirely dissociate the two parts played by the proteins and the inorganic ions respectively, or to say how much, for any given alteration under pathological conditions, may be due to a change in the proteins in cells or plasma, or to a change in inorganic ions.

In order that the relationship of the observed decrease in acid secreting power of the gastric glands in carcinoma, to changes in the plasma may be understood, it may be pointed out that the plasma is a fluid in which normally the concentrations of hydrogen and hydroxyl ions are never so widely different as in dilute solutions of free acid or alkali, so that in this respect the plasma approximates in chemical reaction to distilled water. This has been shown by a number of observers<sup>1</sup> by direct measurements of the hydrogen ion concentration by means of hydrogen electrodes. So near does the reaction of the plasma lie to that of distilled water that the variations in the concentration of the hydrogen ions cannot be estimated by physical methods, which are only capable of showing that the plasma never becomes strongly acid or strongly alkaline.

Although the constituents of the plasma are so balanced that the concentrations of hydrogen or hydroxyl ions lie much closer together than in even a very dilute solution of a free acid or alkali, such as a  $\frac{1}{1,000}$  normal solution for example; yet the behaviour of the plasma towards acids or alkalies is entirely different from a fluid neutral in the sense that distilled water is neutral. The difference lies in the fact that the plasma, on the addition of an acid or an alkali, is much more stable in the concentration of its hydrogen and hydroxyl ions than is the water, because of the fact that the plasma contains substances capable of acting either as acids or bases.

The understanding of this difference in behaviour of two fluids, which are both practically neutral in reaction, is of fundamental importance not only in regard to the immediate problem of the absence of hydrochloric acid in the gastric juice in cancer, but also in regard to the life of the cell and its reaction to changes in its environment.

1. Fraenkel, Arch. f.d. ges. Physiol. vol. 96, 1903, p. 601.  
1. Höber, Ibid., vol. 99, 1903, p. 572.  
1. Farkas, Ibid., vol. 98, 1903, p. 352.

Without a fluid, in which the reaction was not only nearly neutral but stable in its neutrality, the life of the cell, as at present constituted, would be impossible. The cell and also the organism of which such cell is a part always reacts in such a way as to preserve this approximate neutrality against any change from without. The plasma with its almost neutral reaction (just on the alkaline side and nothing more than this) is a special mechanism provided to protect the cells against any sudden or excessive change in the relative concentration of hydrogen and hydroxyl ions, or in other words, against variations in chemical reaction.

Without such a protective action, the cell would at once be killed by the acid products of its own metabolism, for the limits of relative concentration of hydrogen and hydroxyl ions, within which life is possible, are very narrow. An excess of either hydrogen or hydroxyl ion at once combines with and fixes the protein of the cell, and by so doing, stops the power of the protein for combining with other substances, metabolism is accordingly abruptly stopped and life becomes impossible.

Thus, as has been shown by Moore, Roaf, and Whitley,<sup>1</sup> addition of as little of either acid or alkali as suffices to produce a solution of  $\frac{1}{1000}$  to  $\frac{1}{500}$  gram molecular acid or alkali,<sup>2</sup> at once kills organisms which have no protective mechanism, and short of these concentrations most profound changes in growth and nuclear division are obtained, which will be referred to later. These results show how exceedingly narrow are the limits of hydrogen and hydroxyl ion concentration, within which, normal metabolism, and even life itself are possible.

On the other hand, in an organism which possesses a protective mechanism, such as the plasma, sufficient amounts of either acid or alkali to produce twentyfold the above concentrations may be added by direct venous injection, with perfect impunity. It is not that the higher organism is able to withstand change in reaction any better, but that it is able to protect itself against such change.<sup>3</sup>

1. *Proc. Roy. Soc., B*, Vol. 77, 1905, p. 102.

2. The minute dosage is perhaps better shown when one states that these strengths correspond approximately to 1 part in 10,000 of hydrochloric acid or sodium hydrate.

3. It is this very protective action which stands in the way of any interference in the reaction of the plasma, by drugging with acid or alkali.

The action of the tissue fluids, and of the fluids of the cell, as a regulating and controlling mechanism upon variations in concentration of hydrogen and hydroxyl ions (*i.e.*, upon acidity or alkalinity) can perhaps best be understood by comparing the effects of addition of small quantities of acid or alkali upon the ionization in the case of distilled water, and of a solution such as the plasma or lymph containing bi-carbonate of sodium with an excess of carbonic acid, a mixture of mono and di-sodic phosphates, and a quantity of protein, capable of playing the rôle of either acid or base.

In distilled water almost all the water molecules are in the non-ionized condition, as shown by the very feeble conducting power of the water, and but a relatively small number of molecules are in the ionized condition as hydrogen and hydroxyl ions. Since only water is present, all the ionized molecules must come from water molecules, and, as each of these yields one  $\text{H}^+$  and one  $\text{HO}^-$  ion, it follows that the numbers of hydrogen and hydroxyl ions must be equal. This is the condition of exact neutrality, and the distilled water is accordingly an exactly neutral solution containing a very low and equal concentration of hydrogen and hydroxyl ions.

If now there be added to the distilled water but a slight trace of a strongly dissociating acid, such, for example, as sufficient hydrochloric acid to make a  $\frac{1}{1000}$  gram molecular solution, then the distribution of the hydrogen and hydroxyl ions becomes an entirely different one. The slight trace of added acid is sufficient to alter the relative concentration of hydrogen and hydroxyl ions, so that instead of the original equality there are now, at least, 10,000 hydrogen ions for one hydroxyl ion. Exactly a similar change, but in an opposite sense, occurs if a trace of a caustic alkali be added instead of the acid, the concentration of the hydroxyl ions becoming more than 10,000 times that of the hydrogen ions.

Two factors are responsible for bringing about this immense swing in the relative concentration of the two ions.

In the first place, the dissociation of the water is very low, while the trace of added acid becomes at once almost completely dissociated or ionized into hydrogen ( $\text{H}^+$ ) and chlorine ( $\text{Cl}^-$ ) ions. As a result,

although the mass of the acid added is small relatively to that of the water, the complete ionization of the acid leads to the concentration of the hydrogen ion being enormously increased relatively to its former value.

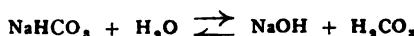
Secondly, the concentration of the hydroxyl ion is depressed in equal degree as the concentration of the hydrogen ion is increased. For the product of the two concentrations of ions remains throughout practically constant, being dependant almost solely on the dissociation factor of the water. If we write  $c_H$  for the concentration of the hydrogen ions, and  $c_{OH}$  for the concentration of the hydroxyl ions, then the law holds that  $c_H \times c_{OH} = k$ , where  $k$  is practically a constant dependant upon the physical characteristics of water. Accordingly, in order that this product may remain constant, if  $c_H$  be increased 100 fold, as it easily is with the small amount of acid we have been considering, then  $c_{OH}$  must be diminished 100 fold. The ratio of  $c_H : c_{OH}$  accordingly becomes 10,000 to 1. But it is the ratio of these two ions upon which the reaction depends, and hence the solution acquires marked acid properties. The change is shown and can be followed quantitatively by the physical methods used for determining ionic concentrations (such as hydrogen electrodes or hydrolysis methods) and it is found experimentally that there is such a rise as described above in hydrogen ions. The same is shown by the behaviour of the very dilute acid solution to coloured indicators ; it is acid to the very strongest of these, such as methyl orange or di-methyl, and reacts positively to Günzburg's reagent. Exactly similar reasoning follows for the addition of a trace of caustic alkali, the hydroxyl ions being here increased and the hydrogen ions depressed.

The case is however quite different even if a considerably greater amount of a bi-carbonate or of a mixture of primary or secondary phosphates, such as are present in blood plasma, be added instead of the acid or caustic alkali. For here, instead of adding to the distilled water a substance which on dissociating yields only one of the two ions concerned in determining the reaction, we are adding substances which, on dissociating, add both ions. Accordingly, the swing in the ratio of concentration of the two ions is very slow, and by adding either

(a) a mixture of sodium bi-carbonate and carbonic acid in proper proportions, or (b) a definite mixture of mono-sodium-phosphate ( $\text{Na}_2\text{HPO}_4$ ) and di-sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), we may obtain a solution in which the relative concentrations of hydrogen and hydroxyl ions do not vary from their values in distilled water. Not only so, but by the presence of such salts in the solution, the concentrations of the two ions are steadied, so that free acid or alkali can now be added or taken away from the solution without causing any such enormous change in relative concentrations as have been shown above to occur in the case of distilled water. This is most important in that it enables the secretion of an alkaline or acid fluid to occur by the secreting cells of the glands of the body, and also renders possible the metabolic processes of the cells.

Taking as the simplest case, the addition of sodium bi-carbonate to water, the processes occurring can be most simply understood if represented as happening in two stages.

First, the molecule of  $\text{NaHCO}_3$  takes up a molecule of water, and partially dissociates into  $\text{NaOH}$  and  $\text{H}_2\text{CO}_3$ , as represented by the equation :—



Next, the molecules of  $\text{NaOH}$  and  $\text{H}_2\text{CO}_3$  ionize, the former yielding ions of  $\text{Na}^+$  and  $\text{OH}^-$ , and the latter ions of  $\text{H}^+$  and  $\text{HCO}_3^-$ .

The final result is that both  $\text{H}^+$  ions and  $\text{OH}^-$  ions are added, and there is not, as in the case of addition of  $\text{NaOH}$ , or of  $\text{H}_2\text{CO}_3$  alone, an addition of  $\text{OH}^-$  ions only, or of  $\text{H}^+$  ions only. Accordingly, the displacement of the ratio of the two ions is much smaller, and in fact, whether the reaction becomes alkaline or acid will depend upon the relative amounts of alkali and carbonic acid.

In dilute solutions of  $\text{NaHCO}_3$ , the reaction is almost neutral ; a trace of  $\text{CO}_2$  bubbled through renders the solution acid, and a trace of  $\text{NaOH}$  in excess gives an alkaline reaction. Exactly similar reasoning holds for a mixture of primary and secondary phosphates, and whether the mixed solution will be acid or alkaline depends on the relative amounts of the two salts.

These conclusions are confirmed both by modern physical methods for determining ionic concentrations and by the effects of the solutions upon coloured indicators.

For example, determinations in the case of the plasma of the hydrogen ion concentration by many observers have, as mentioned above, shown that this lies around the same limits as in the case of distilled water.

Again, although all the coloured indicators used in acidimetry and alkalimetry change colour at extremely low concentrations of acid or alkali (usually much less than  $\frac{1}{10,000}$  gram molecular concentration of free acid or free alkali), yet the blood plasma is alkaline to some of these and acid to others ; thus showing that its own concentrations in hydrogen and hydroxyl ions lie intermediate between the low values at which changes occur to these indicators. Thus blood is acid to phenol-phthalëin and alkaline to di-methyl, but less than  $\frac{1}{5,000}$  gram. molecular caustic alkali in distilled water will turn phenol-phthalëin acid, and less than that concentration of hydrochloric acid in distilled water will turn di-methyl acid. It follows that the reaction of the plasma must lie well within these two very narrow limits of alkalinity or acidity. The difference between the blood plasma and distilled water with regard to what might perhaps be termed its 'reactivity,' since it cannot, properly speaking, be termed its *reaction*, is seen on the addition to the plasma of alkali or acid.

Although the plasma, as shown above, is so near the neutral point, it behaves in a striking manner to alkalies and acids in that it is able practically to neutralize either of these without large changes in the ratio of its own hydrogen and hydroxyl ion concentration, that is to say in its reaction.

The recent results of Moore and Wilson<sup>1</sup> show that before the plasma becomes neutral in its reaction to phenol-phthalëin enough *alkali* must be added to it to correspond to  $\frac{1}{20}$ th to  $\frac{1}{40}$ th gram molecular solution ; while in order to neutralize it to di-methyl enough *acid* must be added to correspond to so high a figure as  $\frac{1}{5}$  to  $\frac{1}{6}$  gram molecular. That is

1. This Journal, Vol. 1, 1906, p. 297.

to say, between these wide limits the relative ionic concentration in hydroxyl ions changes very slowly compared with the changes in presence of alkali or acid.

Let us now turn to the practical application of these considerations to the secretion of the hydrochloric acid by the gastric cells and to the cause of its suppression in carcinoma, and to the effects upon the cells of the body of slight variations (such as can occur within the limits of life) in concentration of hydrogen and hydroxyl ions under pathological conditions.

To make the matter clearer, let us suppose that the plasma instead of having its balancing supply of carbonates, phosphates, and protein substances capable of acting either as weak acids or weak bases, is devoid of this, and consists of a solution of neutral saline, such as sodium chloride. Suppose the gastric gland cells commence to remove hydrochloric acid from such a saline solution, then the merest trace of removed acid with the accompanying removal of hydrogen ions, would cause at once an immense swing up in alkalinity of the plasma, because the ionization of the water of the saline solution is so low. The result would be either that acid secretion must at once stop or the body cells be killed by the alkali which would be absorbed by the proteins of the cells and stop all activity. Further, the merest trace of carbonic acid formed in any act of metabolism by any cell in the absence of alkaline carbonates or phosphates would lead to rise of hydrogen ion concentration and cause the death of the cell.

In the normal process of acid secretion by the gastric cells, such a rise in alkalinity of the plasma is enormously diminished by a slight change in dissociation of the carbonates and phosphates of the alkalies present in the plasma. As a result of such readjustments in dissociation, considerable amounts of acid can be secreted from the plasma without any marked change in the ratio of concentrations of hydrogen and hydroxyl ions, such as would injure the cells.

The work of the acid secreting cells of the stomach then consists in removing the hydrogen ions from the plasma at the exceedingly low concentration at which these ions exist in that fluid and concentrating the solution in hydrogen ions to the value which they possess in the

gastric secretion. Much as in the case of the kidney, the secreting cells of that gland take out urea at the low concentration of urea molecules found in the plasma, and concentrate the solution until the urea molecules possess the concentration found in the urine.

It is clear, therefore, that the rate at which the acid secreting cells will be able to secrete acid, other things being equal, will depend upon the concentration of the hydrogen ions in the plasma. For the lower the concentration of hydrogen ions in the plasma, the greater will be the amount of concentration necessary to produce an acid secretion of a given concentration in hydrogen ions, and the greater will have to be the amount of work done by the plasma in order to produce the secretion.

Now, although as shown above, the variations in ratio of hydrogen and hydroxyl ion concentration in the plasma are small compared to those in a solution containing only free acid or free alkali, where the concentration of the one ion may exceed that of the other by many thousands of times, yet the variations within the narrower limits possible may be very considerable. Thus the determinations of the hydrogen ion concentrations by means of hydrogen electrodes, show variations of about one hundredfold in the hydrogen concentrations.

The position of affairs may be summarized as follows :—

1. Even a very dilute solution of free alkali or acid (such as  $\frac{1}{1000}$  gram molecular solution) leads to an enormous change in ratio of hydrogen and hydroxyl ions (10,000 : 1), which, if it were allowed to occur would instantly lead to the death of the cells.
2. The cells are protected against any such great change by the alkaline carbonates and phosphates.
3. Within much narrower limits (say 100 : 1) changes in ratio of hydrogen and hydroxyl ions can occur, due to alterations in the distribution of carbonates and phosphates, and also probably of the basic and acidic affinities of the circulating and tissue proteins.
4. The rate of secretion of hydrochloric acid, other things being equal, will be directly proportional to the concentration of hydrogen ions in the plasma. Accordingly, the rate of secretion may vary enormously although to ordinary titration methods there appear to be only slight variations in alkalinity of the plasma.<sup>1</sup>

1. The variations in the hydrogen ion concentration of the plasma, which are capable of producing the observed differences in rate of secretion of acid, lie also outside the limits of accuracy of any of the physical methods of determining hydrogen ion concentration, since two readings in the same solution may easily vary by  $0.5 \times 10^{-3} = 50$  fold.

According to the relative distribution of sodium carbonate and carbonic acid, and of the different alkaline phosphates in the plasma, and also of variations in the acidic power of the proteins of the plasma, the concentration of the hydrogen ion may therefore vary within limits capable of effecting very considerably the rate of secretion of the hydrochloric acid, although such variations are small, compared to the variations occurring in a solution containing only free acid or alkali.

The reduced secretion of acid which occurs in cancer points accordingly to an alteration in the plasma from the normal, as a result of which the hydrogen ion concentration is diminished.

Observations by Moore and Wilson<sup>1</sup> of the alkalinity of the inorganic constituents of the plasma have given the result that in carcinoma there does occur a small but definite increase in the alkalinity.

Whether the organic constituents of the plasma and also the constituents of the tissue cells are similarly affected in cancer, is a question urgently requiring attention, but the experimental difficulties of such an enquiry are very considerable.

An increase in the alkalinity of the circulating fluid cannot leave the tissue cells unaffected, for experiment shows how extremely sensitive living cells are to even slight changes in concentration of hydrogen and hydroxyl ions.

An experimental enquiry into the effects of a small chronic change in degree of alkalinity upon living organisms is most difficult to carry out, and one rendered almost impossible in all higher organisms by the adjusting changes in the opposite direction made by the cells of the organism.

Thus, if acids be administered, the cells form ammonia or organic bases to neutralize the acid, and if alkalies be administered they are neutralized by excess of carbonic acid or by organic acid formed by the cells of the organism. In addition, the acid or alkali is removed as rapidly as possible by the excretory cells of the organism, and the amount capable of administration is also limited by the irritability

1. *Loc. cit.*, p. 317

of stomach and intestine, leading to the rejection of the drugs by vomiting, or the appearance of a profuse diarrhoea by which they are carried off without absorption.

A more acute acid or alkaline intoxication can be obtained by intravenous injection, but this does not properly imitate the desired condition.

It is accordingly only in simpler organisms, where a complete protective mechanism does not exist, that experimental results can be obtained.

The fertilized egg of *Echinus esculentus* has been used by Moore, Roaf, and Whitley<sup>1</sup> for studying the effects of acids and alkalies upon cell growth, and division of the nucleus, and their experiments have shown that minute traces of added alkali or acid produce marked and characteristic results.

As stated above, the limits of acidity and alkalinity within which life is possible were found to be very narrow, cell division being entirely stopped, and the cells killed by so low concentrations as  $\frac{1}{1000}$  to  $\frac{1}{500}$  gram molecular acid or alkali added to sea-water. In the still more dilute solutions, in which growth and cell division still continued, it was found that characteristic differences existed between the action of acid and alkali respectively.

The acid solutions caused at no dilution an increase in rate of growth or a stimulus to nuclear division. From the beginning the growth was slowed, mitotic figures were less frequent, and the chromatin showed signs of disintegration and stained more feebly with chromatin stains.

On the other hand, the more dilute alkaline solutions produced a marked favouring action upon cell growth, the organisms developing in these solutions being more advanced in cell division than the controls. Also at a certain level of concentration the growth became most irregular, so that the organisms became misshapen, and the cells irregular in shape and size. The division of the nucleus also was more strongly stimulated than that of the cytoplasm, so that many of

1. Proc. Roy. Soc. B. vol. 77, 1905, p. 102.

the cells became multinucleated. The character of the nuclear division was also changed, so that many tri- and multipolar spindles were formed, and the distribution of chromosomes to the poles became unequal, leading to the formation of unequal sized nuclei. The length of the chromatin rods was altered, so that these appeared shortened and thickened upon the spindle as rounded vesicles which were less in number than the normal.

It is hence clear that a slight increase in hydroxyl ion concentration leads to a marked stimulation of nuclear division, and to abnormalities in the process, some of which strongly suggest the irregularities often described in the dividing cells of malignant growths.

One of the most important facts which has been noted with regard to the local situation of primary malignant growths is that such growths often occur in situations which are liable to or have been the seat of chronic inflammation and irritation. It may be pointed out in relationship to the views expressed above, that such situations would be chronically exposed to a higher alkalinity than other parts of the body.

It has been shown that passing carbon dioxide into blood causes alkali to leave the corpuscles, and so increases the alkalinity of the plasma ; as a result, the alkalinity of the plasma of venous blood is always higher than that of arterial blood, and the lymph of parts subjected to venous congestion is therefore of higher alkalinity than the lymph of parts with a normal circulation.

The experiments mentioned above show that such increased alkalinity will cause a tendency to increased cell growth, and such cell growth within normal bounds is often seen in inflamed or injured situations as a reparatory or protective process. When the irritation and inflammation are long continued, this stimulus by the increased alkalinity to growth and cell division may ultimately lead to malignant growth in such a situation, and the highly venous lymph coming from such a congested area would also act as an irritant to cells along its course.

## SUMMARY

1. The secretion of hydrochloric acid in the gastric juice is very sensitive to any variation in the general health of the body, any enfeeblement in general condition leading to decreased percentage of acid. (Contrast Tables IV and V).

2. The reduction in acid secreting power is much more marked in cancer than in other conditions, free acid being entirely absent in about two-thirds of the cases, and much reduced in most of the remainder. (See averages given on p. 275).

3. The absence of acid or reduction of acid secreting power is independent of the situation of the growth, being seen in cases of cancer of all regions.

4. This points to the change being due to a change in the concentration in hydrogen ions in the plasma and lymph, which may be due to alterations in the inorganic salts or to changes in the organic constituents capable of acting as acids or bases.

5. The alkalinity of the inorganic constituents of the plasma is increased in cancer (Moore and Wilson).

6. Increase in alkalinity up to a given degree leads to increased cell growth and pathological nuclear divisions in organisms which are unable to protect themselves against artificially increased alkalinity (Moore, Roaf, and Whitley).

7. Irritation and inflammation are accompanied by increased alkalinity of the lymph, which is a factor in stimulating cell growth and when long continued may lead to the excessive nuclear division and cell growth of malignancy.

**A CLINICAL METHOD OF HAEMALKALIMETRY, WITH APPLICATIONS TO DETERMINATION OF THE REACTIVITY OF THE INORGANIC SALTS OF THE SERUM IN MALIGNANT DISEASE AND OTHER CONDITIONS**

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The experiments recorded in this paper were commenced with the object of determining the alkalinity of the blood in malignant disease.

Our attention was attracted to this subject in consequence of the observation that secretion of hydrochloric acid in the gastric juice is diminished or suppressed whatever be the situation of the malignant growth, which points to the probability of an increased alkalinity of the plasma in malignancy.<sup>1</sup>

The determination of the reaction of the blood and its degree of alkalinity or acidity, is a subject surrounded by great difficulties, both experimental and theoretical, as is shown by the great number of methods which have been introduced for the purpose, and the great divergencies in the results obtained.

The experimental difficulties are introduced by the colour and composite nature of the fluid, the presence of the proteins, which react as acids or bases, according to the indicator used to point out the neutral point, and the peculiarly balanced phosphates and carbonates of the alkali metals present which act in a similar manner.

The older experimental results vary according to whether the reaction is taken in the whole blood or in the plasma or serum, and still more according to the indicator used by different observers.

1. This Journal, preceding paper, p. 274

The theoretical difficulties are caused by a general haziness, which surrounds the literature of the subject as to what constitutes a neutral reaction in a fluid. It is only recently that advances in physical chemistry and improvements in methods of determining ionic concentrations have begun to shed light on the results of determinations, by means of coloured indicators, of what was formerly supposed to be the alkalinity or acidity, and to show that the reaction lies not very far removed from the neutral point.

It by no means follows from this, however, that titrations with coloured indicators are valueless, and should be abandoned in favour of such measurements of ionic concentrations as can at present be carried out by physical methods.

In the first place, although the titrations do not give the true neutral point, and therefore the degree of alkalinity or acidity of the blood or serum, they do give something which is of high value in physiology and pathology, namely the amount of alkali on the one hand, and acid on the other, which can be added to these fluids without raising the hydroxyl ion concentration or hydrogen ion concentration above certain low limiting values.

This property which is related to the reaction, and yet, in value may be quite different from the reaction, requires a special term, and has been called, in the previous paper, the *reactivity*.

As has been pointed out in that paper, a long range of reactivity is essential to the normal physiological life of the cell, once its limits have been passed, striking pathological changes occur, and with a slight further increase in either hydroxyl or hydrogen ion concentration, life becomes impossible.

The peculiar content of the plasma, and of the cell protoplasm also, in proteins, and in carbonates and phosphates of the alkali metals, increases the range of reactivity, or in other words, the amount of alkali or acid which can be set free in the body without causing the hydroxyl or hydrogen ion concentration to rise so high as to disturb or prevent the metabolic processes in the protoplasm which are necessary for life.

Further, the limits of variation in the ratio of the concentrations of the hydrogen and hydroxyl ions which are compatible with life at all, and certainly with life under physiological conditions, are so narrow as to render the variations in these concentrations impossible of determination by any of the methods at present known in physical chemistry.

For example, the blood plasma or serum gives the acid indication to the indicator, phenol-phthalëin, and the alkaline indication to the indicator methyl-orange. Now if either sufficient alkali were added to give the alkaline indication to phenol-phthalëin or sufficient acid to give the acid indication to methyl-orange, then the evidence of experiment on the effect of such changes upon living cells leads us to conclude that life, as a result of such changes, would become quite pathological if not altogether impossible.

Thus, in the case of the developing fertilized egg of *Echinus esculentus*, it has been shown that a concentration of  $\frac{1}{750}$  normal of either acid or alkali completely stops growth and cell division, and quickly kills the cells.

This illustrates the narrowness of the limits of change in hydrogen and hydroxyl ionic concentrations which are compatible with life, and the necessity of a fluid constituted like the plasma, for preserving the concentrations within physiological limits. Without such a conserving agency any small liberation of acid or alkali in the body would lead to profound disturbances of the metabolic processes.

It is just within these narrow limits of variation in the relative values of hydrogen and hydroxyl ionic concentrations lying around equality, that physical methods of determining ionic concentrations at present leave us without assistance, and where it is, therefore, of value to retain titration methods when read in the light of the knowledge given by the physical methods. For although the titration methods do not give the ionic concentrations, if it can be taken as approximate that the fluid contains carbonates, phosphates, etc., in about the same relative proportions in different cases, then the titrations will be roughly proportional amongst themselves to the ionic concentrations, because they indicate the total concentration in these salts, and upon this the hydrogen and hydroxyl ionic concentrations ultimately depend.

With such an imperfect method we are, at any rate, forced to be content until increased sharpness can be given to physical methods for determining hydrogen and hydroxyl concentrations in such a fluid as blood plasma or serum.

For example, it is quite impossible to determine the concentration of hydroxyl or hydrogen ions in the plasma by the hydrolysis method, and with hydrogen electrodes it is impossible to get any nearer than that the concentration of the hydrogen ions approximates to their concentration in distilled water. Now the results of different observers vary by  $0.5 \times 10^2$  for the value of the hydrogen ion concentration. This means that the concentration of the hydrogen ions might be fifty times that of the hydroxyl ions or, conversely, the concentration of the hydroxyl ions fifty times that of the hydrogen ions without the possibility of detecting a difference by the method. The living cell is so extremely susceptible to variations that it is just in this immeasurable zone that all the physiological interest lies.

Although the recent observations show that the reaction of the serum lies, approximately, at the neutral point are of high interest, such knowledge alone is insufficient, and what is wanted is accurate experimental knowledge of the small amount by which the reaction normally differs from neutrality, and of the small variations which occur in diseased conditions.

The only course, at present available, is titration under uniform conditions, as free from experimental objections as possible.

For this reason we have carried out titrations of the serum to (a) phenol-phthaléin and (b) di-methyl-amido-azo-benzol (called afterwards 'di-methyl' for brevity); and also titrations of the inorganic salts of the serum after removal of the proteins by incineration. Such determinations have been made (a) in healthy individuals of both sexes, (b) in persons debilitated by various diseases or by accidents, and (c) in persons suffering from malignant disease.

Before passing, however, to a description of our methods and the results of our experiments, it will add to clearness to make some preliminary statements as to neutrality to different indicators, its meaning, and its relationship to neutrality as indicated by physical methods.

The statement is often made that the blood (or plasma or lymph) is a fluid which is at the same time acid and alkaline, or at one time the acidity of the blood is spoken of, and at another its alkalinity.

Such statements are, however, meaningless and absurd, the blood cannot at the same time be both alkaline and acid, for the two properties are diametrical opposites of each other. Between acidity and alkalinity there exists a neutral point, and any given solution *must* lie to one side or other of the neutral point, that is to say, it must be either acid or alkaline.

The statements arise from the fact that many fluids (including the blood and other body fluids) show the acid colour or indication to one coloured indicator and the alkaline indication to another indicator.

The statement would, therefore, be somewhat less objectionable that a given solution is acid or alkaline to a stated indicator; for example, the blood is acid to phenol-phthalëin, and alkaline to litmus, methyl-orange and 'di-methyl,' or the urine is acid to phenol-phthalëin and litmus, and alkaline to methyl-orange and 'di-methyl.'

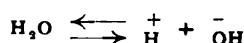
Even this method has the objection, however, that it introduces the conception that one and the same solution has several different neutral points, and that, therefore, the neutral point is a variable thing dependent upon an artificial colouring agent which is merely introduced to discover or show a supposed neutral point existing in the solution before its addition.

The questions may be raised—what is the meaning of all these different neutral points, is there any single definite neutral point not dependant upon indicators, if so, has it any relationship to the various neutral points given by coloured indicators, and, finally, what physiological interest do the reactions to coloured indicators possess?

All acid solutions contain hydrogen ions in excess, and all alkaline solutions contain hydroxyl ions in excess, which at once suggests that the true neutral point between acid and alkali is that point at which the concentrations in the solution of hydrogen and hydroxyl ions are equal.

This condition is realized in pure distilled water, for here the only source of either hydrogen or hydroxyl ions is the dissociation or ionization of the water molecules themselves, and hence the number of the hydrogen and hydroxyl ions must be equal.

The condition of equilibrium in the water may be represented by the equation :—



The excessively low conductivity of pure distilled water shows that the number of conducting ions is very low compared to the non-ionized or water molecules.

If now to the water there be added a *trace* of acid, this is almost completely ionized, and one of the ions is always a hydrogen ion, as a result of this (remembering that the water is practically non-ionized and the added acid almost completely ionized) the hydrogen ion concentration is enormously increased. Also the hydroxyl ion concentration falls in the same ratio, so that the hydroxyl ion concentration is almost reduced to zero. This follows because the *product* of the hydrogen and hydroxyl ion concentrations remains practically constant, and, therefore, as hydrogen ion concentration goes up, hydroxyl ion concentration correspondingly falls, so as to keep the product constant.<sup>1</sup>

As a result of these two factors acting in the same direction it follows that the ratio of hydrogen ion concentration to hydroxyl ion concentration, even in a very dilute solution of an acid, goes up enormously. Similarly in a very dilute solution of a caustic alkali the hydroxyl ion concentration rises and the hydrogen ion concentration falls so that the ratio becomes very high.

As a result even a very dilute solution of free acid or alkali shows most markedly the corresponding acid or alkaline properties. The position is quite different when to the distilled water are added such salts as the acid carbonates and phosphates. Here the ratio of hydrogen and hydroxyl ions varies incomparably more slowly, and,

1. If  $\text{C}_{\text{H}_2\text{O}}$ ,  $\text{C}_{\text{H}}$ , and  $\text{C}_{\text{OH}}$  represent concentrations, then from the equation of equilibrium of the water and its two ions given in the text, we have, from the mass action law, the equation  $K_1 \text{C}_{\text{H}_2\text{O}} = K_2 \text{C}_{\text{H}} \times \text{C}_{\text{OH}}$ , where  $K_1$  and  $K_2$  are two constants, but  $\text{C}_{\text{H}_2\text{O}}$ , the concentration of the water-molecules for dilute solutions is almost constant also, from which it follows that  $\text{C}_{\text{H}} \times \text{C}_{\text{OH}}$  is approximately constant.

according to the relative proportion of acid and base, the hydrogen or hydroxyl ions respectively may be in excess.<sup>1</sup>

The cause of this is that the added acid salts such say as  $\text{NaHCO}_3$ ,  $\text{Na}_2\text{HPO}_4$  and  $\text{Na}_3\text{HPO}_4$  yield on hydrolysis by the water and ionization both hydrogen and hydroxyl ions and not one of these only, so that there is no union of two factors running one concentration up and the other down as in the previous case, but two opposing tendencies which partially balance each other and come into equilibrium.<sup>2</sup>

It is for this reason that the dual reaction to different indicators shown so markedly by blood serum (and many others of the body fluids) arises, and this is also why such a dual reaction is practically never seen with solutions of free acid or alkali even at extreme dilutions.

To understand how this dual reaction arises, and why as a result the same fluid appears to show just as many different apparent neutral points as indicators are used for its titration we must consider briefly what coloured indicators are and how they act. We shall see that they really do not indicate the neutral point but rather a point at which there is a certain definite degree of dissociation or ionization of the indicator itself that this point corresponds to a *comparatively* low ratio of hydrogen and hydroxyl ions, but a ratio which is different for each particular indicator. Also, because the ratio of the hydrogen and hydroxyl ions alters very slowly in a solution of bi-carbonates or phosphates the neutral points to the several indicators are drawn widely apart; while because the ratio varies enormously rapidly when free acid or free alkali is being titrated the neutral points to the different indicators are crowded together so that practically they come to the same point as far as can be experimentally observed, and thus the solution does not indicate acidity to one indicator and alkalinity to another.

The coloured indicators used for titration in alkalimetry consist of organic substances which possess the property of acting as very weak acids or very weak bases, or of salts of such feeble organic acids or bases, further, in solution these become partially ionized and the organic ion resulting from their ionization is coloured and has a different

1. It is not meant by this that the two concentrations are nearly equal; the ratio may be 100 to 1, or 1000 to 1, while with an equal amount of added free acid or alkali it might be 100,000 to 1 or 1,000,000 to 1.

2. See previous paper.

colour from the non-ionized molecule of the salt or organic base or acid which is usually also coloured.

The so-called neutral point is that point at which the concentrations of organic ion and of organic non-ionized substance, in the form of salt, acid or base, as the case may be, are so balanced that the colour is the colour of passage between the colour of the ion and the colour of the non-ionized molecules of the indicator.

The neutral point is hence given by an equality in physiological intensity of two different colour tones blending into the intermediate colour, and it is important that the two colours shall be as distinct as possible so that the physiological effect of the change from the one to the other shall be striking and easily visible. One interesting result of this is that the neutral point varies for different individuals somewhat, so that a series of titrations to be comparable should be made by the same observer, in the same kind of light, and the observer's colour sensations should be sharp and as normal as possible.

It is clear from the above alone that the so-called neutrality of a solution need not coincide with the point at which the concentrations of hydrogen and hydroxyl ions are equal.

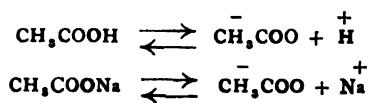
As a matter of fact the point of neutral colour *never* lies at the point of such ionic equality, the ratio of concentration of hydrogen and hydroxyl ion concentrations varies for each indicator, and in the case of any indicator varies also within narrower limits with the concentration of the indicator in the solution, so that it becomes important in any series of titrations to keep the concentration of the indicator constant (compare series below), and also to use the smallest possible concentration of indicator compatible with a clearly observable change in colour.

The ratio of concentrations of hydrogen and hydroxyl ions at the neutral point of colour is always however, a relatively low one compared to that obtaining in even a very dilute solution of any strongly ionized free acid or alkali, and it is upon this fact that the use of indicators for titrating acids or alkalies depends.

The changes in colour in a solution containing an indicator caused by addition of alkali or acid are due to the fact that the

ionization of the salts of these weak organic acids or bases, are different from the ionization of the free organic acid or free organic base. Suppose the indicator is a weak organic acid (as is the case, for example, with phenol-phthalëin or litmus), then addition of excess of alkali will cause the formation of the salt of this acid which is largely dissociated in solution into its ions hence the solution will have the colour of the organic ion (blue in case of litmus, pink in case of phenol-phthalëin). If, on the other hand, acid be added in excess to the solution containing the indicator then the organic acid is set free in the solution instead of the salt, now this organic acid is scarcely dissociated, hence the concentration of the organic ion falls almost to zero and the concentration in molecules of non-ionized indicator rises. As a result the colour of the solution now becomes that of the non-ionized indicator molecules (red in the case of litmus, colourless in the case of phenol-phthalëin). Intermediate between these two points of acidity and alkalinity, there is a point where the two colours of ionized and non-ionized indicator molecules are equal in intensity (or in cases of one colour only where the colour just appears or just disappears) this is the point of neutral colour to the indicator—the so-called neutral point—but it is obvious such a point need not lie at equality in concentrations of hydrogen and hydroxyl ions. Its exact point depends upon those ratios in hydrogen and hydroxyl ions, which, at the given concentration of indicator in the solution, keep it dissociated to that point at which coloured ion and coloured non-ionized molecule of the indicator balance in physiological intensity of colour.

The above reasoning may be made clear by reference to a weak organic acid and one of its salts which are not coloured, but in which the change in ionization can be shown by other physical methods. As such an example we may take a dilute solution of acetic acid and compare it with a solution of sodium acetate of equal molecular strength. In such solutions there is an equilibrium between the ions and the non-ionized molecules which may be expressed by the equations :—



As shown by the electrical conductivity and other physical methods, there is a great quantitative difference in the ionization in the two solutions, for the acetic acid is almost all non-ionized, that is in the condition shown on the left hand side of the equation, while the sodium acetate is strongly ionized and is hence chiefly in the condition shown by the right hand side of the second equation. Suppose now the non-ionized acetic acid ( $\text{CH}_3\text{COOH}$ ) or its sodium salt ( $\text{CH}_3\text{COONa}$ ) had, say a blue colour, and the acetyl ion ( $\text{CH}_3\text{COO}^-$ ) had a red colour, then when in the condition of acetic acid with low ionization the colour would be blue, with scarcely any red which would be concealed by the excess of blue, and if alkali were added it would pass finally into red as the sodium acetate became more and more ionized giving more and more of the red coloured acetyl ion. Conversely, if the solution at first contained highly ionized sodium acetate it would be red in colour, the relatively highly concentrated acetyl red coloured ion out-balancing the small amount of non-ionized blue coloured sodium acetate, and if any acid were added, acetic acid would be formed, which, being but feebly ionized, would give the blue colour of the non-ionized molecules present in excess, and intermediately the neutral tint of purple would be passed through.

It is clear that the neutral point with indicators corresponds to equal physiological intensity in the two colours which are blending, this in turn depends on a certain definite ratio in the concentrations of coloured ion and coloured non-ionized molecule. There need not be equality, in the physical sense, of these two concentrations, and still less need there be equality in the concentrations of hydrogen and hydroxyl ions producing that given ratio of dissociation in the indicator which is responsible for the colour balance.

The ratio in the hydrogen and hydroxyl ions at the point of balance in colour is dependent upon the ease of ionization of the indicator, and this in turn upon the chemical constitution of the indicator and its concentration in the solution.

The same reasoning applies to a coloured base or its salt used as an indicator, except that here the feebly ionized free base is formed by addition of an alkali, while the strongly ionized salt is formed on

addition of an acid. But as before the non-ionized molecule of the indicator must be different in colour from the organic ion of the indicator.

In order that a colour base or colour acid may make a good indicator, it is necessary that the neutral colour points shall correspond to a low ratio in hydrogen and hydroxyl ions, or otherwise it will only react at all with strongly ionized acids or alkalies, and also that the degree of ionization of the indicator shall change rapidly with the change in ratio of hydrogen and hydroxyl ions at the neutral colour point otherwise the colour change will not be sharp.

The sharpness of colour change also depends upon the ionization of the alkali or acid being added, for the more completely ionized this is the less of it will have to be added to cause sufficient change in hydrogen and hydroxyl ion concentrations to induce perceptible changes in ionization of the indicator and so change the colour.

It is clear that an indicator which has its neutral colour point at a low concentration in hydrogen ions is most suitable for titrating weak acids, such as carbonic acid and the higher fatty acids (oleic, palmitic, etc.), for such an indicator will change at an exceedingly low concentration in hydrogen ions. Such an indicator is phenol-phthaléin, and it is for this reason that serum gives an acid reaction to it on account of the excess of carbonic acid which the serum contains. On the other hand, indicators which have their neutral colour point at a comparatively high concentration in hydrogen ions, such as methyl-orange and di-methyl-amido-azo-benzol, do not react at all to carbonic acid and other acids of low ionization, because the concentration in hydrogen ion produced by such acids is never high enough to produce the neutral colour point. It is just such indicators which react best with weak bases, because their neutral colour point corresponds to comparatively high hydrogen ion concentration and, therefore, low hydroxyl ion concentration, and hence in presence of weak bases the colour reaction remains on the alkaline side until a sufficient amount of strongly ionized acid, such as a mineral acid, is added to first combine with the weak base and then rapidly raise the hydrogen ion concentration until the neutral colour point is reached. It is for this reason that

serum is strongly alkaline to methyl orange or 'di-methyl,' and require enough acid to be added to satisfy the combining power of the proteins of the plasma, which act as feeble bases, before the colour changes to show an acid reaction.

Before passing from this brief outline of the modern theory of indicators, in so far as it concerns the interpretation of our results, it may be of interest to point out, since it has also a bearing upon our results, that the proteins of the cell protoplasm, behave to changes in hydrogen and hydroxyl ions in a manner exactly similar to indicators. They are, indeed, substances with feeble alkaline or acid properties which are much more sensitive to changes in hydrogen and hydroxyl ions than are most of the coloured indicators, and all that has been said above about indicators applies equally to them, and is on this account of physiological interest.

The changes produced by alterations in concentration in hydrogen and hydroxyl ions in the living cell are not shown as in the case of indicators by obvious colour changes, but they are none the less obvious, being shown by marked alterations in metabolism, growth, and nuclear division.

The cell is constituted so that it can only carry out its functions, normally, in presence of certain concentrations in hydrogen and hydroxyl ions lying not far removed from equality. It is provided against any marked alteration by a nutrient fluid which prevents sudden changes in the concentrations of these ions, and the composition of the nutrient fluid in this respect is kept delicately regulated by the excretory mechanisms and compensatory metabolic processes, so that it is exceedingly difficult, in the more highly organized animal, to cause, by artificial means, changes in the hydrogen and hydroxyl concentrations to which the cells are subjected.

To summarize, determinations by physical methods of the hydrogen ion concentration in blood or blood serum show that the hydrogen and hydroxyl ion concentrations lie comparatively near together, but such physical methods are not sufficiently delicate to give the small variations of the ratio in hydrogen and hydroxyl ion concentrations in this region, or to demonstrate such alterations in the

ratio as may occur in diseased conditions. Treatment of cells growing in an isolated condition, where there is no compensatory mechanism, to small variations in hydrogen and hydroxyl ion concentrations shows that these are most susceptible to such variations. Although titrations of the serum do not directly give variations in the ratio of the ionic concentrations, they do show the range of acid or alkali which can be added without the respective concentrations passing certain limits ; they also show, at least qualitatively, by increased or diminished titration values that the ratio has changed, and give a rough quantitative index to the amount of change.

For these reasons we have carried out the series of experiments given below, and since other work, already mentioned, had led to the view that alterations might be expected in malignant disease, we have given special attention to determinations of the alkalinity of the serum in a series of malignant cases.

The serum has been used in preference to the whole blood, because, as pointed out by Wright,<sup>1</sup> it possesses the following advantages :—(1) The red blood cells do not interfere, and (2) from a clinical point of view the alkalinity of the serum is more important, because it comes into such close contact with the tissues, and may be taken as an index to the changes taking place in the circulating blood.

It may be pointed out, however, that in the second of the two methods, described below, in which the alkalinity is determined after removal of organic matter by incineration, the whole blood can be used, and we have found by experiment that accurate results can be obtained by the method when the whole blood is taken.

At the outset of the experiments the entire method described by Wright was employed, using litmus paper as an indicator, but it was soon found that reliable results could not be obtained with litmus, because four or five consecutive dilutions of normal acid gave the same colour reaction before a definite acid reaction was reached. This colour varied from pale blue on the outer edge of the moist circle of

1. *Lancet*, 1897, Vol. II, p. 719.

paper made by the drop to pink in the centre, so that it was difficult to decide which dilution corresponded to the neutral point.

Moreover, in addition to these experimental disadvantages, neutrality to litmus does not correspond to any definite point in the titration of carbonates and phosphates, giving neutrality with carbonates when there is a certain excess of carbonic acid above that necessary to form bi-carbonate, and neutrality with phosphates, at an intermediate point between the primary phosphate ( $MH_2PO_4$ ) and the secondary phosphate ( $M_2HPO_4$ ).

The indicators phenol-phthalëin and di-methyl-amido-azo-benzol were, therefore, substituted for litmus. The former of these gives neutrality with carbonates almost exactly at the point where bi-carbonate alone is present, and with phosphates at the point where secondary phosphate ( $M_2HPO_4$ ) alone is present; while 'di-methyl' changes colour at the point where all carbonate is neutralized, and where phosphate is present as primary phosphate ( $MH_2PO_4$ ). The use of these two indicators, therefore, correspond to fairly well-marked points. The colour change to both these indicators is much better marked than to litmus, so that sharper readings can be obtained.

For obtaining the serum, measuring its quantity, and for the process of neutralizing it, the method of Wright has been closely followed.

In the case of the 'di-methyl' titrations a very interesting and high value was obtained for the 'reactivity,' which showed that the proteins of the serum acted as bases, and were completely combined with acid before this indicator reacted. The figure obtained for the reactivity of normal serum was 0.170 to 0.180 normal, this indicates in addition to the reactivity of the inorganic salts, the reactivity of the proteins or in other words their power of combining with acids. The reactivity of the inorganic salts alone after the removal of the proteids was found to be 0.030 to 0.038. Subtracting these figures from the above values for reactivity of proteins, plus inorganic salt, the value of the reactivity for proteins alone is obtained as 0.140 to 0.142 normal.

It may be pointed out how close this value lies to the concentration in entire inorganic salts of the serum as shown by ash analyses or depression of freezing point. The value of the concentration of the total inorganic salts of mammalian serum is equivalent to that of a 0.9 per cent. solution of sodium chloride, this expressed as a fraction of a normal solution amounts to 0.15 normal. It looks from these results as if the content of the normal serum in inorganic salts corresponded exactly to the combining power of its protein constituents, thus indicating that these salts exist in feeble chemical combination or adsorption with the proteins.

On account of the above influence of the proteins in affecting the reactivity of the serum to 'di-methyl,' and also because the degree of reactivity to these so preponderated over the reactivity of the inorganic ions, in a second series of determinations the reactivity of the inorganic salts of the serum was determined after removal of the proteins by incineration.

This method in which only inorganic salts were present was found to give very sharp results.

### FIRST METHOD REACTIVITY OF ENTIRE SERUM

#### TECHNIQUE

*Collection of Blood.*—A finger is first cleansed with methylated ether and 5 per cent. solution of formol, and then pricked with a lancet or needle. By tying a bandage or handkerchief round the wrist the superficial veins are compressed and sufficient blood may be obtained to fill one of Wright's glass capsules.

The blood is collected in these capsules by means of gravity and capillarity, or, if necessary, by suction at the other end of the tube. The ends of the capsule are then sealed in the flame of a spirit lamp or Bunsen burner, and the tube left suspended for twenty-four hours. During this time the serum separates out and, by centrifugalization, may be obtained quite free from red cells.

*Preparations of Acid Solutions and Indicator.*—Normal sulphuric acid was taken as the standard acid for titration. On trial, the reactivity of normal serum to 'di-methyl' was found to be equivalent to 5.5-fold dilution of normal sulphuric acid. A series of dilutions of the normal acid was then prepared, which ranged from a 3-fold to a 8-fold dilution of acid. To every 100 c.c. of these dilutions were added eight drops of 1 per cent. alcoholic solution of 'di-methyl.' This was found to be the least amount of indicator required to give the necessary colour change clearly.

As mentioned above, within certain limits, the degree of alkalinity obtained varies with the amount of indicator used. For this reason the same amount of indicator was always added to the acid.

When required for use a small quantity of mixed acid and indicator was poured into a watch glass.

*Titration of Serum against the Standard Acid Solutions.*—The top of the glass capsule may be easily broken off by first making a nick with a file. A capillary pipette is inserted into the serum and the latter allowed to flow into the tube for a distance of 2 cm. The serum is now allowed to run up the capillary tube for a short distance in order to introduce an air bubble, and a mark is then made at the top of the column of serum with a blue pencil. The end of the pipette is now inserted into the solution of acid against which it is to be titrated, and the acid allowed to run in until the lower end of the serum column is level with the blue mark. In this way an equal quantity of serum and of an acid of known strength is obtained. The contents of the capillary tube are blown out on to a clean white slab and thoroughly mixed with the end of the pipette and aspirated in and out of the capillary tube several times. If the red colour of the acid is not turned yellow a fresh titration is carried out with a weaker acid solution until all red disappears. The first dilution which causes a yellow colour is taken as the index of reactivity. Similarly, if at the first trial a yellow colour is produced, a stronger acid is taken until an orange red is given. The dilution which gives the last yellow colour is the index.

*Preparation of Alkaline Solutions and Indicator.*—For estimating the reactivity of the serum to acid in presence of phenol-phthalæin, dilutions of normal sodium hydroxide solution were used. Solutions were prepared ranging from 20-fold to 40-fold dilution.

On adding phenol-phthalæin to these alkaline solutions it was found that the colour faded in the course of a day or two. On this account when required for use 1 c.c. of the particular solution was put in a watch-glass, and a drop of 1 per cent. alcoholic solution of phenol-phthalæin added. The rest of the technique is as was described for titration against a standard acid solution.

If the pink colour given by phenol-phthalæin with the alkali does not disappear, a weaker alkaline solution is taken, and the solution at which the pink first disappears is regarded as the degree of reactivity. If at the first trial all colour disappears a stronger solution is taken, until a solution is reached which gives a faint pink colour; the solution before this is regarded as the index.

It may be here mentioned that, owing to the alkali in glass, all capsules and capillary tubes were filled with strong hydrochloric acid, and washed through several times with distilled water, and finally dried in the oven at 120° C. in order to drive off any residual acid, after this treatment distilled water left in tubes for twenty-four hours gave no acid or alkaline reaction.

## CLINICAL RESULTS WITH FIRST METHOD

## ENTIRE SERUM

For obtaining the normal reactivity, samples of blood were taken from the workers in the Thompson Yates and Johnston Laboratories of the University of Liverpool. Care was taken to avoid the times during which the reactivity is said to vary, viz., after food and after severe physical exertion. Blood was always taken from these cases at a stated time—12 noon—so as to avoid the effects of breakfast on the alkalinity.

In the case of the cancer patients and the disease controls, the blood was taken in the morning between 9.30 and 10 a.m.

TABLE I  
REACTIVITY OF NORMAL BLOOD SERUM

No. of Case	Sex	Age	Alkalinity to di-methyl expressed as a fraction of normal	Acidity to phenol-phthalein expressed as fraction of normal
I	M	28	0.182 normal	0.033 "
II	M	25	0.166 "	0.036 "
III	M	38	0.166 "	0.028 "
IV	M	23	0.182 "	0.033 "
V	M	30	0.182 "	0.033 "
VI	F	25	0.182 "	0.030 "
Average			0.176	0.032

TABLE II  
REACTIVITY OF BLOOD SERUM IN MALIGNANT DISEASE

No. of case	Sex	Age	Disease and region	Alkalinity to dimethyl expressed as a fraction of normal	Acidity to phenolphthalein expressed as a fraction of normal
I	M	65	Epithelioma of mouth	0.166	0.028
II	M	65	Carcinoma of stomach	0.182	0.033
III	M	55	Epithelioma of cheek	0.200	0.034
IV	M	64	Sarcoma of neck	0.250	0.033
V	M	74	Carcinoma of tonsil	0.200	0.028
VI	M	59	Epithelioma of hand	0.200	0.034
VII	M	28	Carcinoma of stomach	0.182	0.030
VIII	M	37	Carcinoma of stomach	0.250	0.036
IX	M	52	Epithelioma of cheek	0.222	0.031
X	M	49	Carcinoma of stomach	0.200	0.028
XI	M	31	Carcinoma of rectum	0.200	0.033
XII	F	65	Carcinoma of liver	0.250	0.036
XIII	F	32	Carcinoma of rectum	0.222	0.033
XIV	F	59	Carcinoma of breast	0.250	0.033
XV	F	85	Epithelioma of hand	0.200	0.031
XVI	F	69	Epithelioma of cheek	0.222	0.040
XVII	F	67	Recurrent carcinoma of breast	0.200	0.030
XVIII	F	47	Carcinoma of colon (colotomy)	0.182	0.034
XIX	F	48	Carcinoma of colon (colotomy)	0.182	0.040
XX	F	10	Sarcoma of leg	0.200	0.033
XXI	F	52	Epithelioma of leg	0.222	0.033
XXII	F	45	Carcinoma of breast	0.200	0.033
XXIII	F	29	Carcinoma of uterus	0.200	0.030
XXIV	F	54	Carcinoma of stomach	0.222	0.034
XXV	F	41	Carcinoma of uterus	0.222	0.028
XXVI	F	27	Sarcoma of neck	0.200	0.033
Average				0.208	0.031

TABLE III  
REACTIVITY OF BLOOD SERUM IN OTHER HOSPITAL CASES  
(NON-MALIGNANT)

No. of case	Sex	Age	Disease and region	Alkalinity to dimethyl expressed as a fraction of normal	Acidity to phenolphthalein expressed as a fraction of normal
I	M	65	Chronic bronchitis	0.182	0.040
II	M	65	Chronic bronchitis	0.200	0.034
III	M	35	Paralysis of legs	0.182	0.030
IV	M	78	Chronic bronchitis	0.182	0.028
V	M	44	Old fracture of thigh	0.222	0.033
VI	M	49	Ulcer of leg	0.200	0.037
VII	M	45	Sprained shoulder	0.182	0.048
VIII	M	56	Wound of scalp	0.166	0.040
IX	M	45	Old hip disease	0.166	0.036
X	M	57	Ulcer of leg	0.182	0.033
XI	M	41	Varicose veins of leg	0.200	0.033
XII	M	43	Abscess of neck	0.166	0.022
XIII	M	31	Tertiary syphilis	0.182	0.033
XIV	M	49	Sprained arm	0.166	0.037
XV	M	45	Ulcers of leg	0.166	0.036
XVI	M	38	Tubercular knee	0.165	0.028
XVII	M	34	Tubercular thigh	0.182	0.033
XVIII	M	19	Tubercular knee	0.182	0.037
XIX	M	47	Tubercular hip	0.200	0.025
XX	M	23	Tubercular pelvis	0.182	0.033
XXI	M	57	Diffuse scaly eczema	0.153	0.025
XXII	F	19	Moist eczema of face and arms	0.182	0.027
Average				0.181	0.033

TABLE IV

## EFFECT OF AN INCREASED AMOUNT OF INDICATOR ON REACTIVITY OF BLOOD SERUM IN MALIGNANT DISEASE

No. of case	Sex	Age	Disease and region	Alkalinity to di-methyl expr. used as a fraction of normal
I	M	79	Rodent ulcer on cheek	0.166 normal
II	M	62	Epithelioma of mouth	0.166 "
III	M	37	Glands in neck secondary to epithelioma of lip	0.166 "
IV	M	38	Epithelioma of lip	0.166 "
V	M	69	Epithelioma of lip	0.153 "
VI	M	61	Sarcoma of jaw	0.166 "
VII	F	36	Carcinoma of uterus	0.166 "
VIII	F	78	Rodent ulcer on nose	0.153 "
IX	F	71	Carcinoma of breast	0.166 "
X	F	40	Carcinoma of uterus	0.153 "
XI	F	67	Sarcoma of neck	0.153 "
XII	F	75	Epithelioma of lip	0.153 "
XIII	F	64	Carcinoma of breast	0.166 "
XIV	F	48	Carcinoma of breast	0.166 "

NOTE.—Normal serum with the same amount of indicator reacted to 0.153 to 0.143 normal.  
The average for the above fourteen cases is 0.161.

## CONSIDERATION OF RESULTS OF FIRST METHOD

On examining these tables it is evident that, as regards acidity to phenol-phthalein, no definite conclusions can be drawn.

It is true that some cases show an increased acidity with a decreased alkalinity, but on the whole no definite relation can be established. On considering the average of the values obtained, it is seen that—

6 healthy cases show an average of 0.032 normal

26 cancer cases     ,     ,     0.031     "

22 cases of other diseases     ,     0.033     "

The alkalinity to 'di-methyl' on the other hand shows some striking results.

The upper limit of alkalinity for healthy subjects is 0.182 normal

Now of 26 cancer cases, 21 are increased, while of 22 control cases of other diseases, which were nearly all at or near the cancer age, only five are above the normal alkalinity.

Average of 6 healthy cases     ...     0.176 normal

   ,     26 cancer cases     ...     0.208     "

   ,     22 cases of other diseases 0.181     "

## SECOND METHOD

## REACTIVITY OF INORGANIC SALTS OF SERUM

## TECHNIQUE

*Collection of Blood.*—The blood is collected in the same manner as in the first method. The capsules are left suspended for twenty-four hours and then centrifuged.

*Standard Acid Solutions and Indicator.*—Dilutions of normal sulphuric acid are prepared commencing with a 28-fold dilution, and advancing by 0.5-fold dilutions to 40-fold.

The indicator used is 'di-methyl.' When an acid solution is required for use, 0.5 c.c. is put in a watch-glass and to it is added the 'di-methyl.' The amount of indicator used in any series of observations should always be the same. In the present investigation the following plan of ensuring this was adopted:—A piece of thick glass tubing of fine calibre, about 20 centimetres in length, was drawn out at one end to a fine point. The rest of the tube was covered with paraffin wax and twenty 1.5 centimetre divisions etched on. The tube was now calibrated by means of mercury, and each centimetre length was found to have a capacity equal to 0.00475 gram of water. Hence each division on the tube corresponded to a capacity of 0.00288 c.c. The tube was filled with di-methyl, and to each 5 c.c. of acid solution was added the contents of three of these divisions. This is a simple way of ensuring that the amounts of acid solution and indicator always bear the same ratio to each other.

*Incineration.*—The serum is allowed to run up a capillary tube for a distance of 6 or 7 centimetres and a mark is made with a blue pencil opposite the top of the column of serum. The serum is now blown out on to a small platinum dish, which is then transferred to a drying oven in order to ensure the complete evaporation of moisture without any spouting. When perfectly dry the platinum dish is placed over a Bunsen flame and all organic matter completely incinerated.

The capillary tube is washed out two or three times with distilled water and the washings transferred to the platinum dish, to be in turn dried and incinerated. In this way all residual serum is got rid of from the tube.

The platinum dishes used are about the size of a penny piece, and in the centre a well is made about the diameter of an ordinary lead pencil and 4 m.m. in depth.

In this well serum and washings are placed, and in it all titrations against the standard acid solutions are made. A simple but quite effective drying oven can be made out of any tin with a hinged lid, such as some tobacco tins, by inserting a shelf of wire gauze.

*Titration against Standard Acid Solutions.*—The acid solution and the indicator are placed in a watch glass, as mentioned above. Another watch glass containing some of the acid solution, but no indicator, is also required. This is for washing out the capillary tube which contained the serum, after it has been washed out with distilled water. The capillary tube is now plunged into the mixed acid and indicator solution

which is allowed to run up to the blue mark. In this way equal amounts of serum and acid solutions are used. The acid is now blown out into the well of the platinum dish which contains the dried inorganic salts of the serum. Titration is effected by means of a small glass rod, through which the colour changes can be very clearly seen.

If after titrating for two or three minutes the pink colour still persists, a fresh titration with a weaker acid solution is undertaken until the colour disappears. The point at which the colour first completely disappears is taken as the neutral point. Similarly if the colour goes at the first trial a stronger acid is taken, until the colour faintly persists. The solution before this point is regarded as the index.

After each titration the platinum dish and the glass rod are thoroughly washed with distilled water.

#### ADVANTAGES OF THE METHOD.

1. Owing to the small amount of apparatus necessary, and the small quantity of blood required, the method may be used clinically.

2. Although serum was used in the research described below in order to compare the results with those obtained by the first method, nevertheless, the total blood may be used as all organic matter is burnt off. This is a great advantage as in all other methods in which the total blood is used the results are obscured by the colouring matter and the great dilution necessary to overcome it. Moreover, as Loewy has pointed out, with these methods, unless the blood be titrated slowly and at the body temperature, all the corpuscles are not broken down, and the total alkalinity is therefore not detected, and that probably, owing to the neglect of this precaution, previous results have given too low values.

3. The colour change is very sharp and clearly defined. The delicacy of the method is proved by the fact that the colour often goes on using a 0.5-fold weaker acid, which contains an amount of acid very little less than the solution which was too strong.

4. Although only small quantities of serum are used, the accuracy of results, judged by controls obtained by using large amounts, is remarkable.

It is important that all glass capsules and capillary tubes should be made free from excess of alkali by the means described under the first method.

#### CLINICAL RESULTS OF SECOND METHOD

##### INORGANIC SALTS OF SERUM

The aims of the research, for which this method was used, were—

1. To establish the normal reactivity of the inorganic salts in the serum, in both men and women.

2. To find out in what ways, if any, these salts varied from the normal in malignant disease.

The blood for these observations in all cases was taken at least three hours after breakfast so as to exclude the possible influence of digestion. None of the subjects had indulged in severe physical exertion on that day.

Some of the cases of malignant diseases had been operated on when their blood was removed.

TABLE V

## BASIC REACTIVITY OF INORGANIC SALTS OF NORMAL BLOOD SERUM

No. of case	Sex	Age	MALES	Alkalinity to di-methyl expressed as a fraction of normal
I	M	29		0.0312 normal
II	M	26		0.0312 "
III	M	23		0.0307 "
IV	M	26		0.0312 "
V	M	24		0.0312 "
V1	M	25		0.0292 "
VII	M	28		0.0307 "
VIII	M	36		0.0307 "
IX	M	25		0.0312 "
X	M	25		0.0303 "
XI	M	39		0.0307 "
XII	M	25		0.0307 "
XIII	M	27		0.0307 "
XIV	M	23		0.0307 "
XV	M	32		0.0303 "
XVI	M	34		0.0312 "
XVII	M	29		0.0292 "
XVIII	M	23		0.0312 "
XIX	M	22		0.0303 "
XX	M	22		0.0294 "
XXI	M	26		0.0303 "
XXII	M	22		0.0312 "

TABLE VI

## BASIC REACTIVITY OF INORGANIC SALTS OF NORMAL BLOOD SERUM

No. of case	Sex	Age	FEMALES	Alkalinity to di-methyl expressed as a fraction of normal
I	F	...		0.0303 normal
II	F	...		0.0292 "
III	F	...		0.0322 "
IV	F	...		0.0307 "
V	F	...		0.0303 "
VI	F	...		0.0322 "
VII	F	...		0.0292 "
VIII	F	...		0.0307 "
IX	F	...		0.0312 "
X	F	...		0.0307 "
XI	F	...		0.0307 "
XII	F	...		0.0292 "
XIII	F	...		0.0303 "
XIV	F	...		0.0312 "
XV	F	...		0.0307 "
XVI	F	...		0.0307 "

TABLE VII

BASIC REACTIVITY OF INORGANIC SALTS OF BLOOD SERUM  
IN MALIGNANT DISEASE

MALES				Alkalinity to di-methyl expressed as a fraction of normal
No. of case	Sex	Age	Disease and region	
I	M	63	Epithelioma of tongue	0.0322 normal
II	M	48	" "	0.0327 "
III	M	63	" "	0.0317 "
IV	M	70	" "	0.0322 "
V	M	63	" "	0.0312 "
VI	M	38	" "	(Recurrent) 0.0317 "
VII	M	54	" "	0.0312 "
VIII	M	43	" "	0.0312 "
IX	M	53	" "	0.0307 "
(Five days after operation).				
X	M	25	Carcinoma of Rectum	0.0327 "
XI	M	61	" "	0.0317 "
XII	M	38	" "	0.0317 "
XIII	M	51	" "	0.0317 "
XIV	M	59	" "	0.0303 "
XV	M	45	" "	0.0327 "
(Fourteen days after operation)				
XVI	M	53	Carcinoma of Rectum	0.0307 "
(16 days after operation)				
XVII	M	58	Rodent ulcer of lips	0.0333 "
XVIII	M	75	" eyelid	0.0322 "
XIX	M	79	" cheek	0.0317 "
XX	M	48	Epithelioma of penis	0.0322 "
XXI	M	47	" "	0.0307 "
(Nine days after operation)				
XXII	M	64	Carcinoma of Oesophagus	0.0327 "
XXIII	M	44	" Colon	0.0327 "
XXIV	M	36	Sarcoma of jaw	0.0312 "
XXV	M	54	" neck	0.0322 "
XXVI	M	18	" testicle	0.0322 "
XXVII	M	57	" neck	0.0333 "

TABLE VIII

BASIC REACTIVITY OF INORGANIC SALTS OF THE BLOOD SERUM  
IN MALIGNANT DISEASE

FEMALES				
No. of case	Sex	Age	Disease and region	Alkalinity to di-methyl expressed as a fraction of normal
I	F	50	Carcinoma of breast	0.0317 normal
II	F	52	" "	0.0333 "
III	F	56	" "	0.0327 "
IV	F	57	" "	0.0307 "
V	F	52	" "	0.0322 "
VI	F	56	" "	0.0307 "
VII	F	37	" "	0.0327 "
VIII	F	58	" "	0.0312 "
IX	F	38	" "	0.0303 "
X	F	50	" "	0.0312 "
XI	F	39	" "	0.0333 "
XII	F	47	" "	0.0312 "
XIII	F	56	" "	0.0302 "
XIV	F	37	" "	0.0327 "
			(7 days after operation)	
XV	F	53	Carcinoma of breast	0.0303 "
			(14 days after operation)	
XVI	F	49	Carcinoma of rectum	0.0312 "
XVII	F	29	" "	0.0317 "
XVIII	F	37	" "	0.0312 "
XIX	F	64	Malignant glands in axilla (2 days after operation)	0.0307 "
XX	F	48	Carcinoma of thyroid gland	0.0322 "
XXI	F	45	Epithelioma of tongue (14 days after operation)	0.0322 "
XXII	F	62	Rodent ulcer of eyelid	0.0322 "
XXIII	F	38	Sarcoma of breast	0.0312 "
XXIV	F	8	" skin of arm	0.0322 "

TABLE IX

## BASIC REACTIVITY OF INORGANIC SALTS OF BLOOD SERUM IN OTHER DISEASES

No. of case	Sex	Age	Disease and region	Alkalinity to di-methyl expressed as a fraction of normal
I	M	34	Septic anaemia	0.0277 normal
II	M	32	Tubercular hip	0.0292 "
III	M	28	" knee	0.0303 "
IV	M	25	" shoulder	0.0312 "
V	M	12	Diabetes	0.0285 "
VI	M	33	"	0.0312 "
VII	M	38	"	0.0303 "
VIII	M	21	"	0.0307 "
IX	M	39	"	0.0289 "
X	F	43	"	0.0307 "
XI	F	30	Eclampsia	0.0303 "
XII	F	62	Tubercular breast	0.0292 "
XIII	F	16	Adenoma of Thyroid gland	0.0292 "
XIV	F	28	Mammary abscess	0.0307 "
XV	F	18	Psoriasis	0.0307 "

TABLE X. AVERAGES

No. of cases	Sex	Condition of health	Average alkalinity to di-methyl expressed as a fraction of normal
22	M	Healthy	0.0304 normal
16	F	"	0.0306 "
38	M + F	"	0.0305 "
27	M	Cancer	0.0318 "
24	F	"	0.0316 "
51	M + F	"	0.0317 "
15	M + F	Diseases other than cancer	0.0297 "

## SUMMARY AND CONCLUSIONS

1. Although the results of physical methods show that the ratio of the concentrations of hydrogen and hydroxyl ions in the plasma is a comparatively low one, thus indicating an approximation to neutrality ; yet the cells are so extremely sensitive, in their metabolism, growth, and nuclear division, to small variations in this ratio, that it becomes essential to study the effects of small variations in the ratio around the neutral points upon the cells.

2. Such small variations cannot at present be followed by any known physical method, and the only approximate method available is that of determining the *reactivity* of the plasma or serum to acids and alkalies by means of indicators.

3. Coloured indicators for acids and alkalies do not show the neutral point of equality in concentration of hydrogen and hydroxyl ions, but a point at which the ratio of the concentrations of these two ions has a certain comparatively *low* value. The value of the ratio at the neutral colour point is dependent upon the ratio of dissociation and association of the indicator, and this in turn upon the nature of the indicator, and secondarily upon the concentration of the indicator. Hence one indicator changes for low hydrogen ionic concentration (such as phenol-phthalein), and another for low hydroxyl ionic concentration (such as di-methyl-amido-azo-benzol). The ratio of the concentrations in the case of serum lies at a low intermediate value, so that it gives the acid colour with phenol-phthalein, and the alkali colour with 'di-methyl.'

4. The resistance of the serum to rapid displacement of the ratio in the two ions when acid or alkali is added may be termed the *reactivity* of the serum. The amount of alkaline reactivity may be determined by titration with acid in presence of a strong indicator such as 'di-methyl' or methyl-orange, which changes with a concentration of hydrogen ions somewhat higher than that of distilled water<sup>(1)</sup>; and the

1. That this range in concentration is not a pronouncedly acid one is shown by the fact that about 1 c.c. of decinormal hydrochloric acid in 1 litre of distilled water (i.e. a  $\frac{1}{1000}$  normal solution) give a distinctly acid reaction with 'di-methyl' or methyl orange.

amount of acidic reactivity by similar titrations with alkali in presence of a weak indicator which changes with a concentration in hydroxyl ions somewhat higher than in distilled water (e.g., phenol-phthalëin).

In all cases the indicator used for determining the reactivity should be stated, e.g., the alkaline reactivity of the entire serum to di-methyl is 0.166 to 0.182 normal, and its acidic reactivity to phenol-phthalëin is 0.028 to 0.036 normal.

5. The long range in reactivity possessed by the serum (and also by the plasma and other body and tissue fluids) is due (*a*) to the inorganic salts such as bi-carbonates and primary and secondary phosphates of the alkali metals which it contains, and (*b*) to the proteins present which are capable of functioning as feebly dissociated acids or bases, and so restraining any great movement of the ionic ratio in either direction outside of physiological limits.

This range of reactivity is essential to the continuance of life, because of the extreme sensitivity of the living cell to variations in the ratio of concentration of the two ions.

6. The susceptibility of the living cell to increase in concentration of either hydrogen or hydroxyl ions is due to the fact that the protein of the cell possesses strong affinity for both these ions forming feebly disassociated salts with them. So that in the presence of higher concentration than the normal of either ion the protein becomes fixed, and no longer possesses that liability of composition and affinity for attaching organic nutrient substances which is essential to its metabolism.<sup>1</sup>

Just as acids and alkalies affect the association or dissociation of coloured indicators and alter their colour by means of the changes which the acids or alkalies cause in concentrations of the two ions, so do acids and alkalies affect living cells and the proteins of the plasma and tissue fluids, and the picture given by the coloured indicators may be regarded as a model illustrating what occurs in the action of acids or alkalies upon living cells.

1. The action of all anaesthetics is similar in character. See Moore and Roaf, *Proc. Roy. Soc.*, Vol. 73, 1904, p. 382; *Ibid.*, B, Vol. 77, 1905, p. 86.

7. The high value for the alkaline reactivity of the proteins of the serum to 'di-methyl' found above (0.15 normal) is of interest, because of its close coincidence with the molecular concentration of the *total* inorganic salts of the serum, as shown by depression of freezing point or incineration methods (0.14 normal), indicating that there probably exists an adsorption or combination between the proteids and the total inorganic salts of the plasma which regulates the total amount of such salts in the plasma.

8. Values have been obtained for the acidic reactivity of the entire serum to phenol-phthalëin, and the basic reactivity to 'di-methyl' in (a) normal individuals, (b) cases of malignant disease, and (c) cases of disease or debility from other causes.

No constant variations have been found in the index of acidic reactivity, the averages running:—0.032 normal; 0.031 cancer; 0.033 other cases. A definite variation of basic reactivity has been found in the direction of increased alkalinity in the malignant cases, the averages running:—0.176 normal; 0.208 cancer; 0.181 other cases.

9. Values have also been obtained for the basic reactivity to 'di-methyl' of the inorganic salts of the serum after removal of the proteins by incineration. Such determinations have been carried out in normal individuals of both sexes, in cases of malignancy in both sexes, and in cases of non-malignant disease or debility.

The results show (a) that there is no appreciable variation with sex either in normal individuals, nor as between the two sexes, in malignancy, and (b) that there is a small but distinct increase in basic reactivity in the malignant cases, which is not shown by the non-malignant cases. The averages run:—Health, 0.0304 (males), 0.0306 (females); malignant cases, 0.0318 (males), 0.0316 (females); non-malignant cases, 0.0297 (males and females).

10. The difference in direction of increased alkalinity in cancer is less after removal of the protein than before its removal, indicating the possibility of the proteins in cancer possessing a higher basic reactivity than normal.

11. Although the differences in basic reactivity between the serum of malignancy and that of normal individuals are small (about 16 per cent. in the whole serum and 4 per cent. in the inorganic salts) yet they are quite definite, and entirely outside the region of experimental error of the methods employed.

The point of neutral colour is particularly sharp in the determinations of the inorganic reactivity, where we feel quite certain we can depend upon the accuracy of each reading. Further, the possibility of error from the minute quantities taken was considered, and duplicates done in the sera of animals, by the clinical method, and by controls on the large scale in platinum vessels. As a result, a very close accord was found between the two sets of results.

With the whole serum the end point is not so sharp on account of the presence of the proteins, but duplicate determinations were always carried out, and the experimental errors possible lie well within the larger difference of 16 per cent. in the two averages.

Also the series of cases observed are of considerable length, and the large preponderance of the malignant cases lying above normal is obvious from the tables.

12. With regard to the important point as to whether this increased basic reactivity in malignancy is to be regarded as a cause or an effect of the disease it may be pointed out (a) that the increased basicity is present in early cases where there could not probably be any large amount of altered material coming from the seat of the growth; (b) that the increased basicity is present after removal of the growth; and (c) the important fact that there is a general concurrence in the results of earlier observers, that there is an increase in alkalinity (reactivity) of the serum in old age when the organism is liable to the onset of malignancy.

## THE PHYSIOLOGICAL ACTION OF ETHYL CHLORIDE, BROMIDE, AND IODIDE, AND OF 'SOMNOFORM'

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*From the Physiological Laboratory, University of Manitoba, Winnipeg, Canada*

*(Received May 21st, 1906)*

Although some of the anaesthetics mentioned in the title, particularly ethyl chloride, have now been in use for some years, and although considerable clinical experience has been accumulated, yet very little experimental pharmacological work seems to have been performed with these drugs. The only papers to which I have found reference are a short note by Cole,<sup>1</sup> and papers by Lebet,<sup>2</sup> and Ginsburg.<sup>3</sup>

Cole's experiments were performed with somnoform and ethyl bromide. He found that somnoform markedly increases the size and rate of contraction and the tone of the diaphragm, which remains in a state of strong tonic contraction, the heart still beating strongly. This effect is obtained after section of the vagi, and is, therefore, due to an action on the respiratory centre. With both somnoform and ethyl bromide small doses cause a rise of blood pressure and acceleration of the heart. With large doses, and especially after section of the vagi, a gradual fall takes place. With intact vagi the heart rate is increased by small doses and diminished by large ones. With vagi cut the usual effect is slowing.

The acceleration with intact vagi Cole attributes to a paralysis of the vagal nerve-endings by the ethyl bromide. He states that during

1. *Proc. Physiol. Soc.*, June 15, 1903 (*Journ. of Physiol.*, Vol. XXIX, p. 25).

2. *Bullet. Acad. de Médecine de Belgique* (4), XV, 5, p. 343.

3. *Inaug. Diss.*, St. Petersburg, 1893. I have not been able to gain access to the communications of Lebet and Ginsburg.

anaesthesia produced by somnoform or the bromide it is impossible to affect the heart by stimulation of the peripheral end of the cut vagus. He further states that it is either the ganglion cells or preganglionic endings which are affected. As will be seen below, I have been unable to confirm these statements.

My thanks are due to Professor Swale Vincent for assistance and criticism during the progress of this research.

#### METHODS AND MATERIAL EMPLOYED

The experiments have been conducted upon forty dogs, two cats, and several frogs. The dogs and cats were anaesthetised, in the first instance, with ether, chloroform, or the A.C.E. mixture, and sometimes, in addition, by the intravenous or subcutaneous administration of morphia. In those animals in which a tracing of auricle and ventricle was taken curari was administered and artificial respiration maintained.

The drugs under investigation were administered by the tracheal tube. Respiration was recorded by means of a Marey's tambour, the volume of a limb, intestinal wall, or spleen by means of a piston recorder.<sup>1</sup> Blood pressure was taken from the carotid artery. A glass plethysmograph was used for recording changes in the volume of the limb, and an air oncometer made of gutta-percha for the intestine. The method described by Oliver and Schäfer<sup>2</sup> was employed for recording the effects upon the heart. A curved metal bar was pressed in the auriculo-ventricular groove, so that the movements of auricle and ventricle might be recorded independently.

The ethyl chloride used was partly that sold under the name of 'narcotile' by Bengué, of Paris, and partly 'kelène' from Fries Bros., of New York. The ethyl bromide was manufactured by Squibb, of New York. The somnoform was that prepared by A. Rousseau, Bordeaux. The ethyl iodide was for some experiments obtained from Kahlbaum, of Berlin, and in others a specimen kindly

1. The instruments employed were obtained from Allbrecht, of Tübingen. It has been found convenient to have the counterpoise of the piston recorder worked on a threaded rod, so as to facilitate accurate adjustment.

2. *Journal of Physiol.*, XVIII, p. 256. 1895

prepared for me by Mr. Thorwaldson in the chemical laboratory of the University of Manitoba.

The ethyl iodide was tested only for the purpose of making the investigation more complete, not with any idea of its being employed generally as an anaesthetic.<sup>1</sup> In order to obtain any marked effect it was necessary to place the bottle containing the drug in hot water in order to vaporise it more rapidly.

It is remarkable how uniform have been the effects of the drugs in different experiments. The results in different individual dogs have indeed varied widely in a quantitative sense, and idiosyncrasy plays a large part in the conditions affecting the action of these anaesthetics in dogs as in the human subject, but qualitatively the results of the administration can be predicted with tolerable certainty.

The differences in action between the chloride, bromide, iodide, and somnoform is one of degree only, and this degree seems to depend simply on the volatility of the drugs.<sup>2</sup> Somnoform is stated by Cole to be a mixture of 65 parts of ethyl chloride, 30 parts of methyl chloride, and 5 parts of ethyl bromide.<sup>3</sup> The presence of methyl chloride does not appear to affect the results.

#### EFFECTS ON THE RESPIRATION

In small doses the rate and depth of respiration are markedly increased immediately on administration, gradually becoming normal after the anaesthetic is removed (See Fig. 1). This occurs in the majority of instances, though sometimes the rapidity is increased while the depth is unaffected, and occasionally the depth is increased while the frequency is unaltered. (See Fig. 2.)

With larger doses the respiration is often temporarily increased, just as with a small dose, but soon the breathing becomes shallower and less frequent. (See Figs. 5, 10, 11, 12.) With still larger doses the respiration stops completely—usually 2-3½ minutes before the heart ceases to beat. (See Figs. 3 and 4.)

1. Although the iodide undoubtedly has anaesthetic properties, yet its lack of volatility (boiling point 72° C.) and its nauseous odour would prevent its use for this purpose in the human subject.

2. The boiling point of the chloride is 12° C., of the bromide 38·4° C., of the iodide 72° C.

3. The proportions are given differently by some writers. Thus McCardie (*Brit. Med. Jour.* March 17, 1906, p. 616) gives it as ethyl chloride 65, methyl chloride 35, and ethyl bromide 5 parts.

4. This fact would appear to be of importance as indicating the value of artificial respiration in cases of an overdose of these drugs. I have in several instances restored an animal by artificial respiration after voluntary respirations had ceased for some time (30-90 seconds), and the blood pressure had fallen almost to zero. (See Fig. 12.)

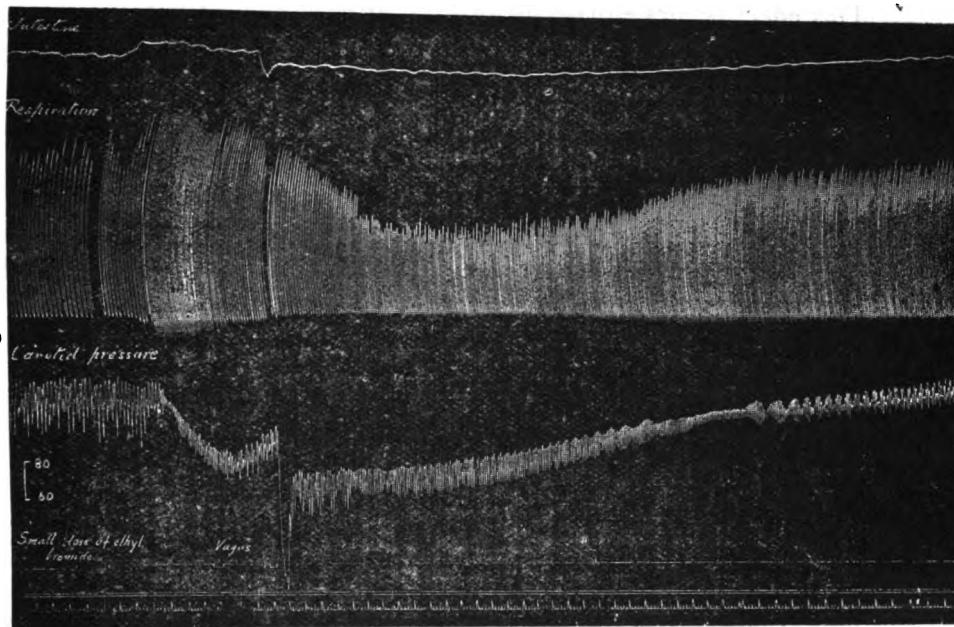


Fig. 1. Dog, 11.7 K. A.C.E.—Ether. The tracing shows the effect of a small dose of ethyl bromide followed by a stimulation of the vagus while the animal is fully under the effect of the anaesthetic. The respiration is at first deepened and increased in rapidity, later becoming shallower, though still frequent.

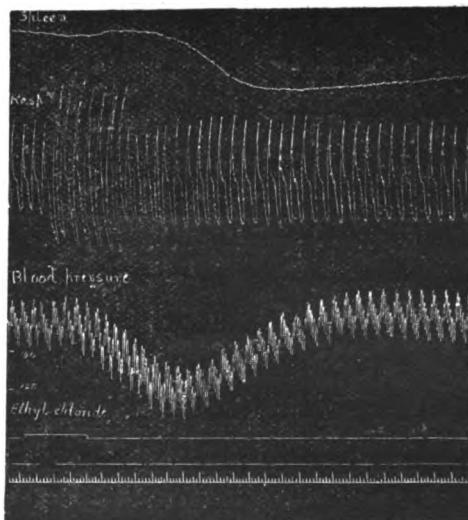


Fig. 2. Ethyl chloride, small dose. Bitch, 8 K. Spleen, respiration, carotid blood pressure. A.C.E., morphia.

This effect occurs equally before and after section of both *vagi* as well as after the administration of nicotine and is probably due, as suggested by Cole, to a direct action on the respiratory centre. Whether the effect is primarily, or indeed chiefly upon the diaphragm as stated by Cole we cannot be certain.

In some instances the animal recovers after respiration has been in abeyance for one minute or even for a longer period, the respiration beginning with very small movements and gradually increasing in extent. (See Fig. 2.) As a rule the recommencing respiratory movements, although shallow, are very frequent. (See Figs. 3, 5, and 7.)

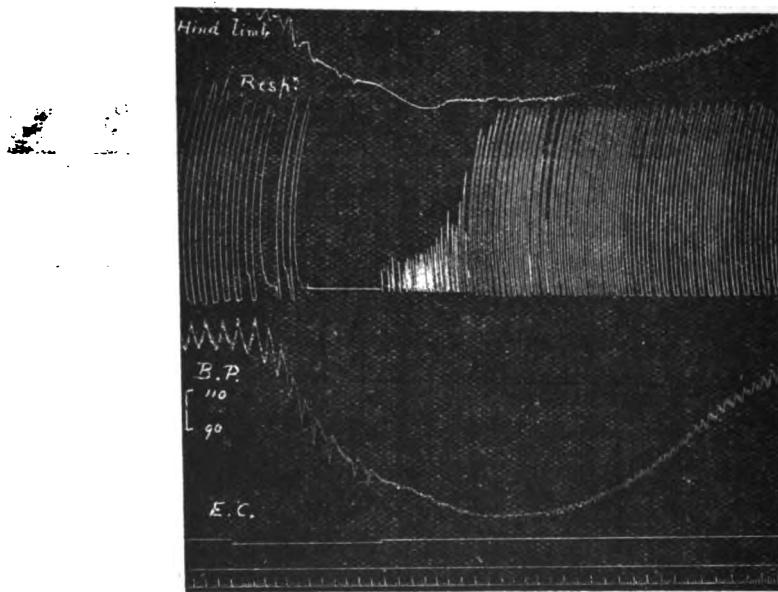


Fig. 3. Hind limb respiration: Carotid blood pressure. Dog, 10 K. A.C.E.—nicotine—both *vagi* cut. Ethyl chloride. Shows sudden stoppage of respiration with recovery. No steps taken to aid recovery. Ether-morphia-curari-nicotine. Both *vagi* cut.

With a fatal dose the respiration, after being stopped for two or more minutes, will usually become re-established for a few seconds before death; stopping finally at the same time as, or a few seconds before the heart. (See Fig. 4.)

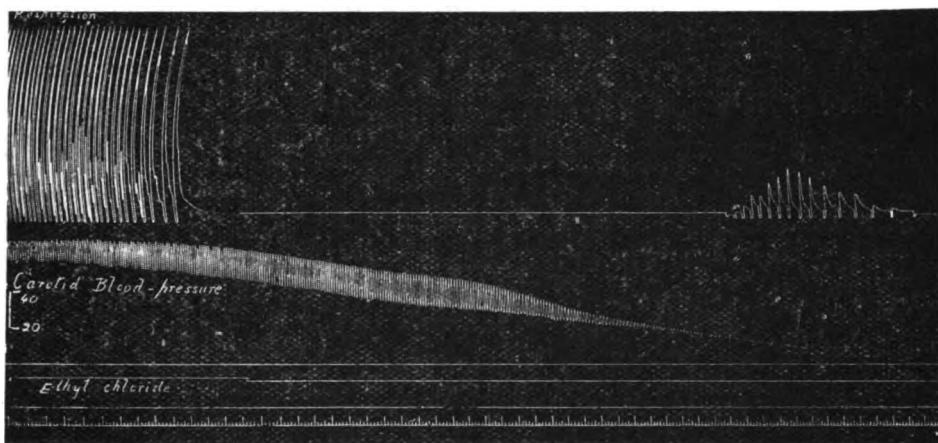


Fig. 4. Dog, 4.8 K. Ethyl chloride, overdose, death. Sudden failure of respiration, interval of three minutes then fifteen respirations occur just before stoppage of heart.

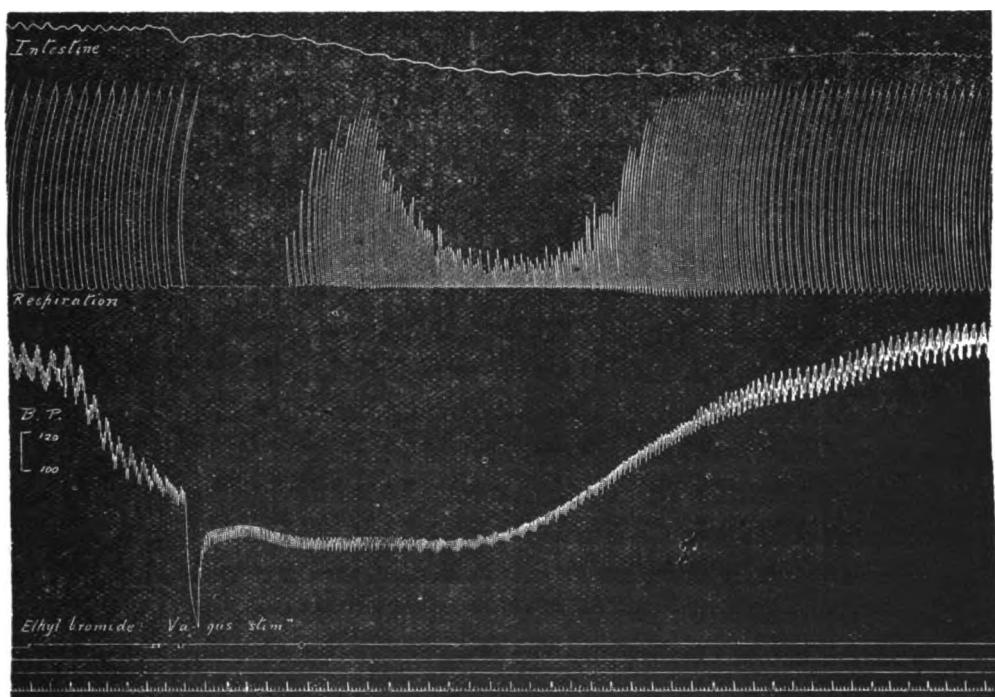


Fig. 5. Intestine, respiration, carotid blood pressure. Dog, 9.6 K. A.C.E.—morphia. Both vagi cut. Ethyl bromide. Stimulation of peripheral end of vagus.

## THE CAROTID BLOOD PRESSURE

With small doses there is generally a slight preliminary rise in blood pressure followed by a return to the normal. (See Fig. 6).<sup>1</sup>

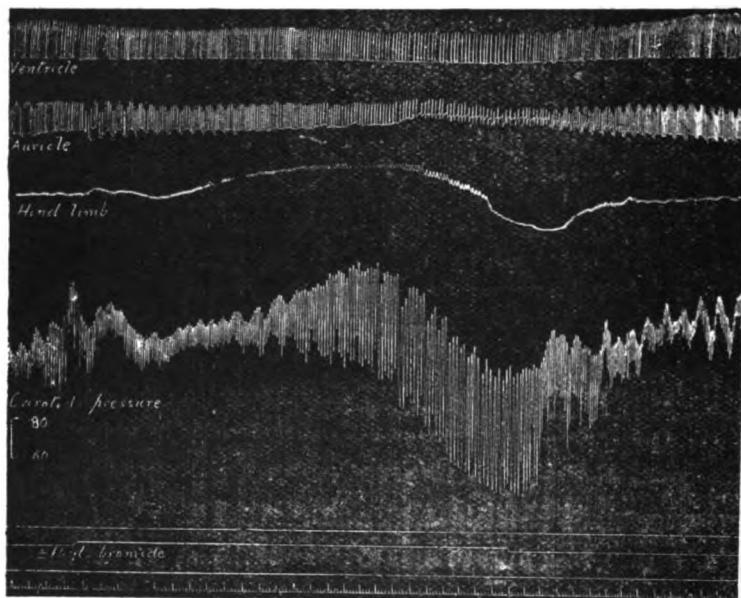


Fig. 6. Ventricle, auricle. Hind limb. Carotid blood pressure. A.C.E. morphia-curari. Ethyl bromide. Small dose at (1), larger dose at (2).

With larger doses the pressure rapidly falls. (In two instances in dogs the heart and respiration have ceased simultaneously). As pointed out by Cole the fall is more pronounced after section of the vagi. The tracings reveal the further fact that after the administration of atropine a more marked effect is liable to occur. In two animals, one of which was being anaesthetised with ethyl chloride, the other with ethyl iodide a rapidly fatal result occurred after atropine with an amount of the anaesthetic which before atropine had only produced a slight effect<sup>2</sup>.

1. In this tracing the pressure falls below normal. Notice also the marked slowing of the heart, as shown both by the blood-pressure tracing, and the record of auricle and ventricle.

2. This would seem to denote that the use of atropine is contraindicated in cases of overdose from these drugs.

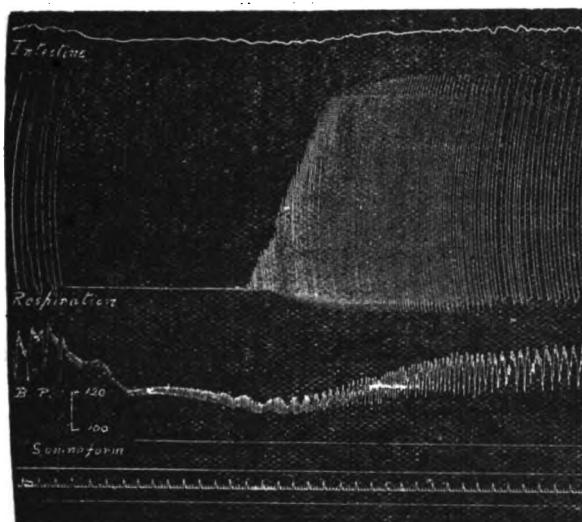


Fig. 7. Intestine, respiration, carotid blood pressure. Dog, 11.7 K. A.C.E.—ether. Both vagi cut. Somnoform.

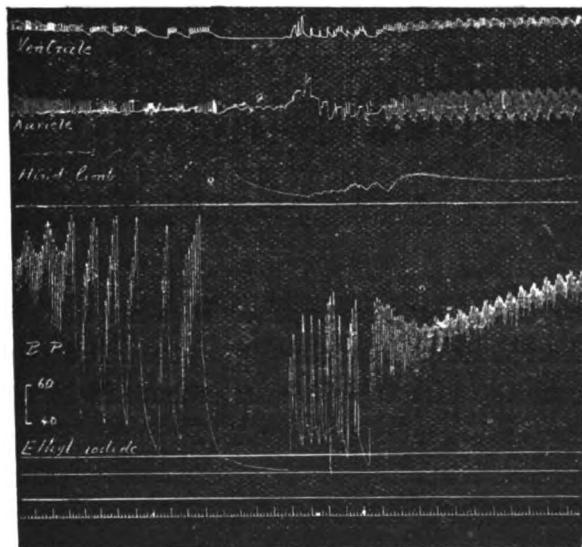


Fig. 8. Ventricle, auricle, hind limb, carotid blood pressure. Dog, 6 K. Ether—morphine—curari. Ethyl iodide, large dose, stoppage of heart, blood pressure falling almost to zero. Recovery without interference.

## THE HEART

With intact vagi the frequency of both auricle and ventricle is sometimes increased, though this does not always occur with small doses. A common result, even with small doses, is the diminution of the frequency and the extent of excursion of both auricle and ventricle (see Fig. 6). I find with Cole that the conduction of the auriculo-ventricular ring is depressed by large doses, the ventricle beating at half the rate of the auricle.

With the vagi cut, the heart is usually rendered less frequent and the excursions of both auricle and ventricle are diminished.

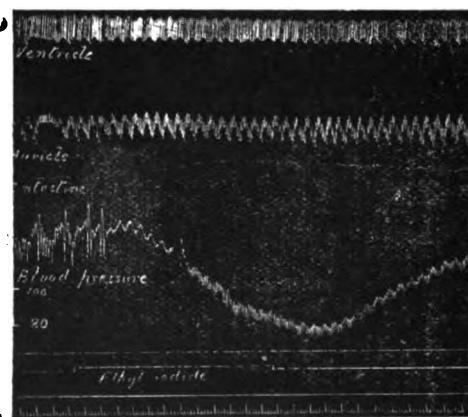


Fig. 9. Dog, 7·4 K. Ventricle, auricle. Intestine, carotid pressure. A.C.E.—morphia—curari. Vagi intact. Ethyl iodide. Shows increased frequency of heart after ethyl iodide.

Ginsburg<sup>1</sup> states that the vagus endings are unaffected by ethyl bromide, and attributes the quickening of the heart to the excitation of the accelerator. Although Cole states that ethyl bromide paralyses the vagal nerve endings, he agrees with Ginsburg that the drug may sometimes have a stimulating action on the accelerators.

I have been quite unable to confirm Cole's observation that ethyl bromide paralyses the vagus terminals, and this applies equally to ethyl chloride, ethyl iodide, and somnoform. Although in about twenty

1. Quoted from Cole.

experiments with the different drugs named, the vagus in the neck (cut or uncut) has been stimulated when the animal was fully under the influence of the anaesthetic, never has there been any diminution of the usual inhibitory effect. Often, on the contrary, the effect of vagus stimulation seems to have been exaggerated. (See Figs. 1 and 5). This point has been further investigated upon the frog's heart recorded by the suspension methods. In two or three experiments it has been impossible to abolish the vagus action on either pre- or post-ganglionic fibres with ethyl bromide. Moreover there cannot be any action on the ganglion cells since the effect can be induced after pouring the drug upon the heart.

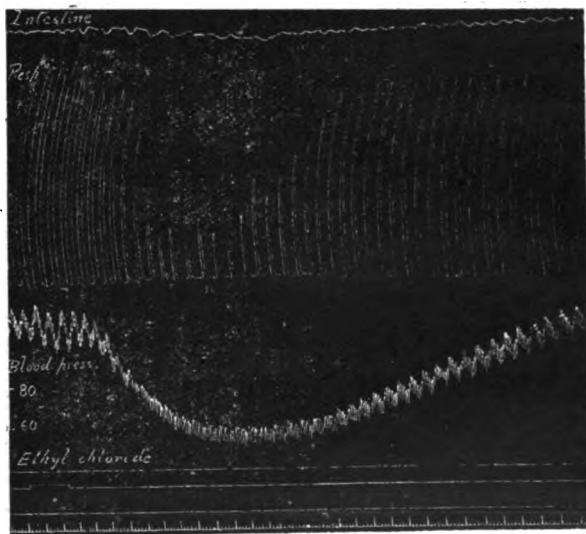


Fig. 10. Intestine, respiration, carotid blood pressure. Dog, 9.6 K. A.C.E.—morphia. Ethyl chloride. Shows diminution in extent and in frequency of respiration with recovery.

#### MODE OF ACTION OF THE DRUGS

The fall of blood-pressure, which always occurs, except perhaps with minimal doses, seems to be due to diminished force and frequency of the heart-beat. On the other hand the slight temporary rise with small doses, or as a first stage with larger doses, is due to a preliminary increase in force and frequency of the heart.

The volume of a limb, of intestinal wall, or of spleen almost always follows passively the blood pressure. During the preliminary rise of blood pressure there is a slight temporary dilatation of these organs, but when the blood pressure has become lowered there

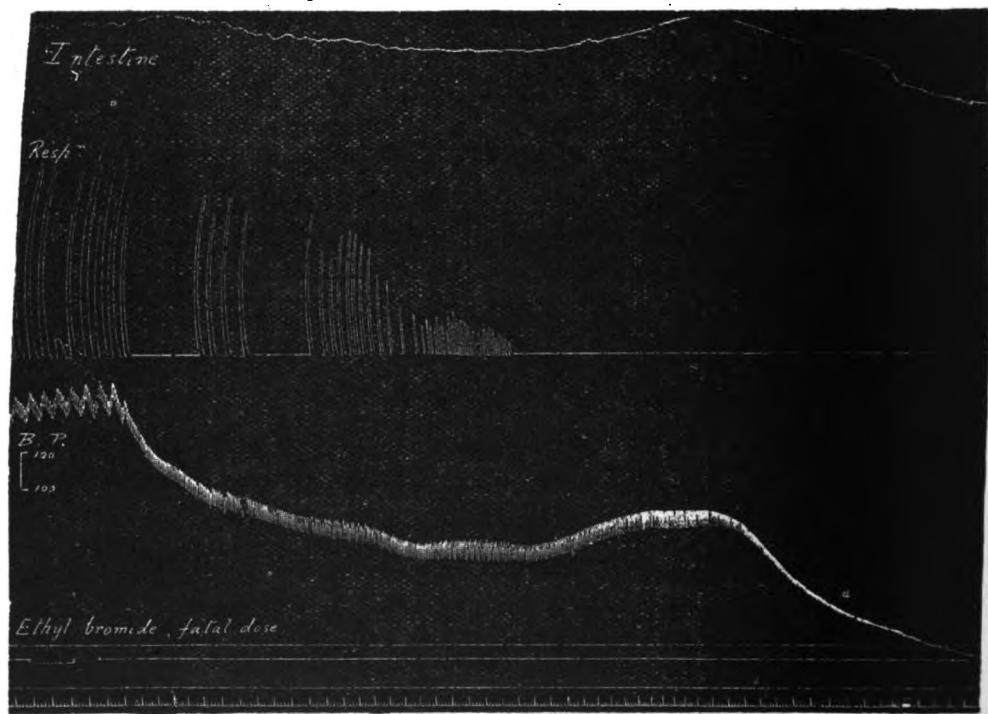


Fig. 11. Dog, 9.6 K. A.C.E.—morphia. Fatal dose of ethyl bromide. Intestine respiration, carotid blood pressure.

is a constriction of these organs. (See Figs. 2, 6, 8, 7, 11). Thus we may say that in most cases the drugs have no effect on the vaso-motor system, or directly on the muscular walls of the peripheral arterioles. The blood is pumped into or drained out of the various organs of the body according to the strength of the heart.

Occasionally, however, one gets the opposite result, though never to a marked degree. Fig. 9 shows a slight dilatation of the intestinal wall after administration of ethyl iodide, and in this instance the dilatation appears to correspond with the fall of blood pressure. My clinical experience, which only extends to ethyl chloride and ethyl bromide, bears out the results obtained by physiological experiment. Clonic spasms occur with about the same frequency in human beings as in

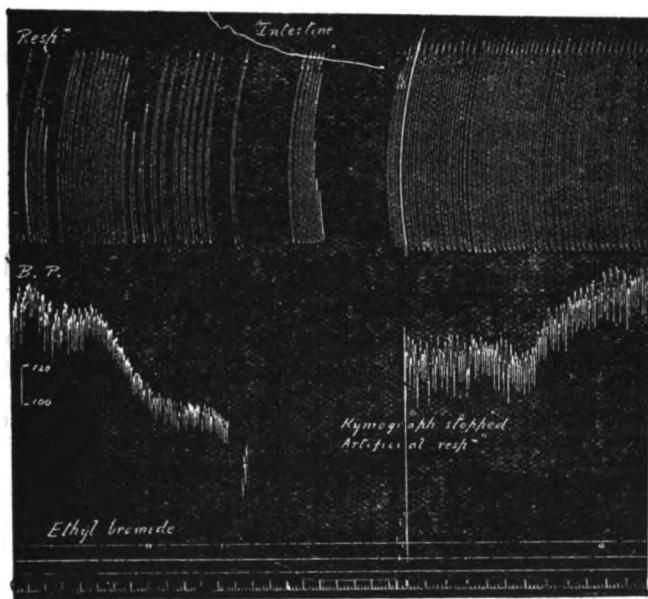


Fig. 12. Dog, 9.6 K. A.C.E.—morphia, ethyl bromide, overdose, blood pressure drops to 2.5 m.m. above zero. Heart and respiration both ceasing. Artificial respiration, recovery.

animals. It is stated by many anaesthetists that 'ethyl chloride is a cardiac stimulant and that if a full dose is administered it dilates the peripheral arterioles.' The stimulating effect upon the heart is at best only slight and transitory, and is followed by a much more significant and lasting depressant effect. As we have seen there is only a passive dilatation of peripheral arterioles, and in full doses the blood is drained from the splanchnic area and also from the limb.

1. Knight, *Brit. Med. Journ.*, March 17, 1906.

## SUMMARY

1. Ethyl chloride, ethyl bromide, ethyl iodide, and somnoform all have practically the same physiological action. Such differences as exist between them are only of degree, and appear to depend on the extent of their volatility.
2. With small doses the respiration is increased in frequency and depth, with larger doses it is diminished in both these respects. With very large doses it may cease entirely some considerable time before the heart stops.
3. With small doses the blood pressure is slightly raised, but with larger doses may be depressed with or without a small preliminary rise. These effects occur with intact vagi, with both vagi cut, after full doses of atropine, and after nicotine.
4. The drugs do not paralyse the vagus nerve-endings. It is possible to obtain full vagus action when the animal is deeply under the influence of the drug.
5. The action of these anaesthetics upon the circulatory system appears to be almost entirely directly upon the heart. The volume of the spleen, intestinal wall, or limb follows passively the change in blood pressure.

## APPENDIX

By SWALE VINCENT, M.B. (LOND.), D.Sc. (EDIN.)

*Professor of Physiology in the University of Manitoba*

Since Dr. Webster handed in his paper to me I have seen Cole's full paper in the *Brit. Med. Journ.*, June 20, 1903.

Cole points out that the chief danger in the use of somnoform being a paralysis of respiration, careful watch must be kept over the respiratory movements. He also notes that after cessation of the respiratory movements it is easy to restore the animal by artificial respiration. This has been fully confirmed by Dr. Webster, and the restoration by artificial respiration is well shown in his tracing, Fig. 12.

But as regards the effects of ethyl bromide and somnoform on the vagus, Webster's results are diametrically opposed to those of Cole. The tracings given by Webster show conclusively that full vagus effects can be obtained when the animal is completely under the influence of the drug.

EDINBURGH

May 17, 1906

## THE EFFECTS OF INJECTION OF ANTITOXIC AND ANTI-BACTERIAL SERA ON THE OPSONIC POWER OF THE BLOOD

By WARRINGTON YORKE, M.B., CH.B. (*Liverpool*), and C. HAROLD SMITH, M.B., CH.B. (*Liverpool*).

(Received May 22nd, 1906)

Last January, owing to an accidental inoculation with a culture of tetanus bacilli, one of us had a prophylactic dose of 10 c.c. of antitetanic serum. As we were working at the opsonic index at the time, it occurred to us that it would be interesting to note the effect, if any, of this injection on the opsonic power of the blood to tetanus bacilli.

Two days elapsed after the injection before the first observation was made, but from this time onwards a daily examination of the blood was undertaken. On the first day, as is shown in the accompanying chart, the tetano-opsonic index was considerably raised. On each succeeding day it fell, and on the sixth day after injection it was found to be below normal (.7). On the eighth day the individual who had the injection of antitetanic serum, whilst using his own blood as a control, discovered that his tuberculo-opsonic power was considerably depressed. An estimation was immediately made, not only with tubercle but also with staphylococci, and his index was found to be low to both organisms. In passing it might be mentioned that before injection his index to tubercle was normal.

To continue—on the tenth day his tetano- and staphylo-opsonic indices were still lower, and it is interesting to note that coincidently with the low opsonic power of the blood, a marked urticarial, and on the following day a purpuric rash broke out all over the body. The examination of the opsonic power of the blood during the following days showed a gradual return to normal. (See Fig. 1).

Having noticed these facts in connection with the injection of antitetanic serum, we then proceeded to see if similar changes would occur with the use of antistreptococcic serum. The streptococcus used for this purpose was obtained from the blood of a patient suffering from malignant endocarditis. After finding that the opsonic

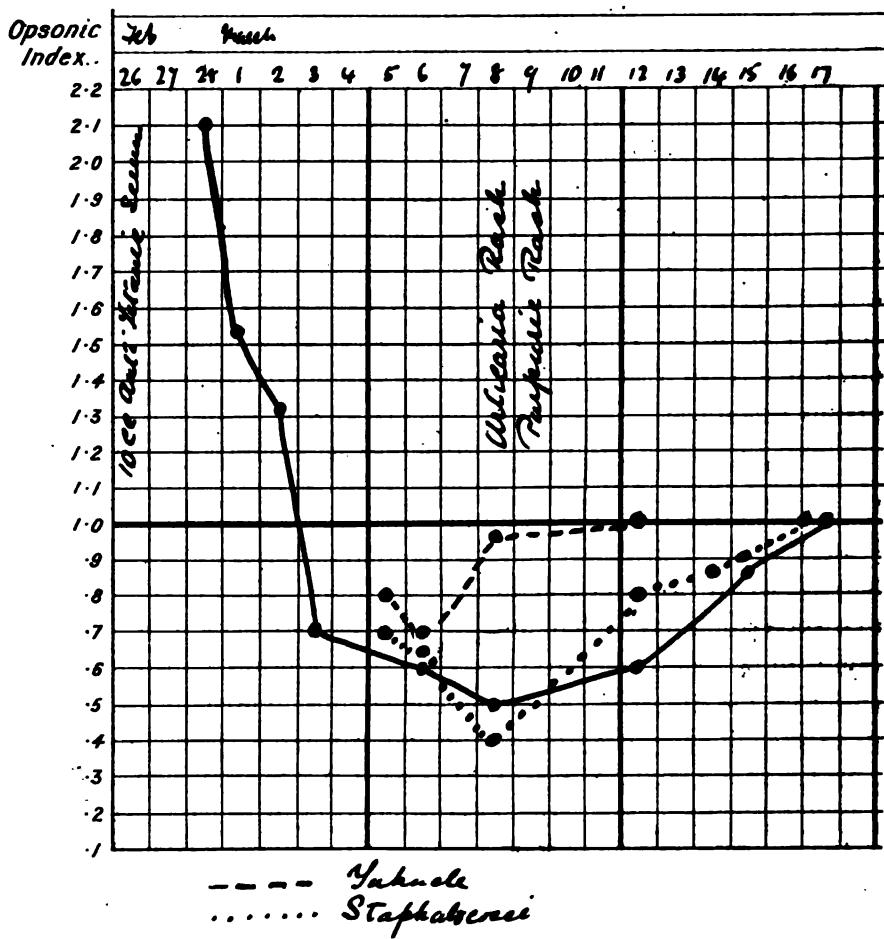


Fig 1.—Chart showing the effect of the injection of 10 c.c. of antitetanic serum on the opsonic index.

index of a man was normal to this streptococcus on three consecutive days, 10 c.c. of antistreptococcic serum was administered subcutaneously. No effect on the opsonic index was noticed until the seventh day, when it had fallen to .5.

The index to *B. coli* which had previously been shown to be normal was now found to be low also. This depression of the index continued until the eleventh day, when on examining the films we found that the washed leucocytes besides containing bacteria had also ingested red blood corpuscles. This phagocytosis of the red blood corpuscles was observed for several weeks. Two or three days later, we incubated for fifteen minutes, equal quantities of washed leucocytes obtained from several individuals, among them from the patient himself, with equal volumes of his (the patient's) serum. In some of these the red corpuscles were noticed inside the leucocytes, in others they were not. There were no red corpuscles observed in the leucocytes obtained from the patient himself.

As in the case of the antitetanic serum certain general effects were noticed, namely :—The patient injected, who was suffering at the time from interstitial nephritis, had a decided increase in the amount of albumin in his urine and developed a little oedema about the same time as the phagocytosis of the red corpuscles was observed. Ordinary blood films made from the patient at the same time shewed nothing abnormal.

As a rise in the tetano-opsonic index occurred after injection of antitetanic serum it may seem strange that there should be no similar rise with antistreptococcal serum, but since there are probably several varieties of streptococci it was thought that possibly the particular strain of streptococcus which was used had not been employed in the preparation of the antistreptococcal serum. In connection with this it might be mentioned that the patient from whose blood the streptococcus used in this experiment was obtained had been treated with the antistreptococcal serum for several days without effect. The experiment therefore was repeated in an exactly similar manner with the exception that the streptococcus used was procured from a case of erysipelas. In this instance there was a definite rise in the index on the fifth day after injection from normal to 1.7 as shewn in the chart. (Fig. 2).

The later effects were very similar to the former, the red blood corpuscles being noted inside the leucocytes about the eleventh day,

though in this case it was not nearly so marked as in the preceding. On referring to the chart it will be noticed that the fall of the opsonic power both to the streptococci and *B. coli* was not so great as in the former instance. This, together with the less marked ingestion of the red blood corpuscles, might possibly be accounted for by the fact that in the latter experiment the person's kidneys were normal, and so the excretion of toxins from the blood would be more readily accomplished.

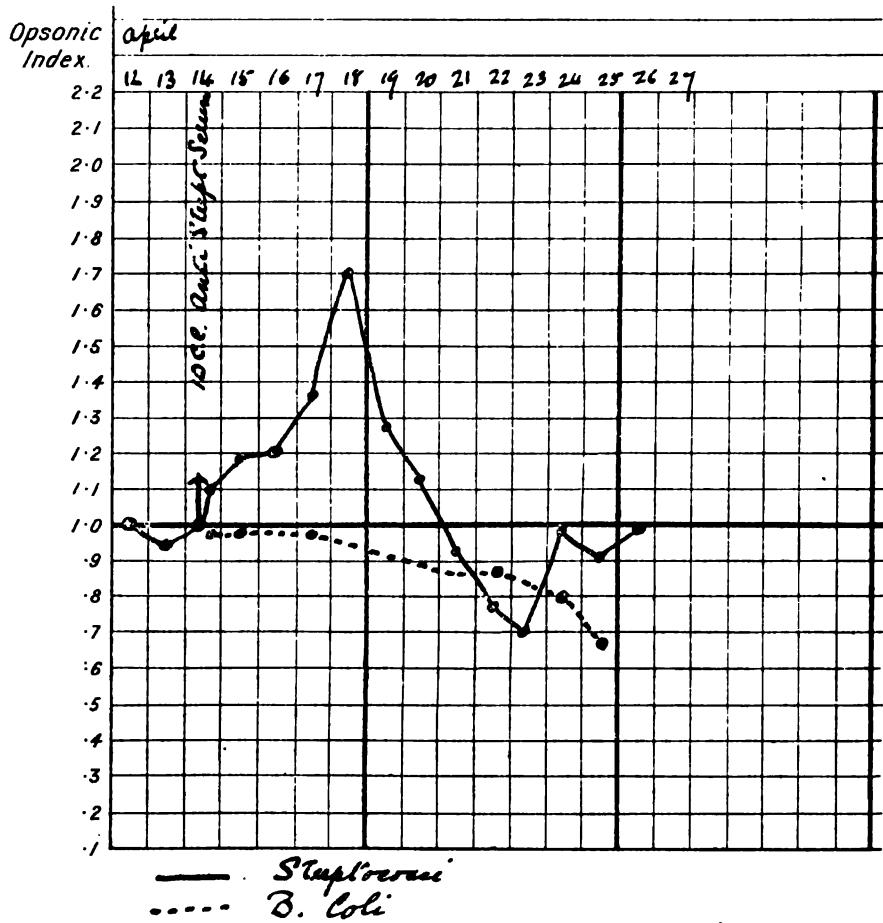


Fig. 2.—Chart showing the effect of the administration of 10 c.c. of anti-streptococcal serum on the opsonic index.

We next attempted to ascertain the effect of antidiphtheritic serum on the opsonic index. After finding that a certain person was normal to the diphtheria bacillus on three consecutive days, 2,000 units of

antidiphtheritic serum was administered. The first result was a fall in the diphthero-opsonic index to .7.

This was followed on the fourth day after injection by a rise which reached 1.6, and finally there was a secondary fall due to the general poisonous effects of the serum appearing about the tenth day.

As in the case of the antitetanic and antistreptococcic serum the opsonic index to other organisms was also lowered.

The serum of the patient mixed with washed corpuscles also produced in a slight degree a phagocytosis of the red blood corpuscles.

Another person was injected and the experiment repeated in an exactly similar manner and substantially the same results were obtained, the only difference being that the rise in the opsonic index was not so marked.

The conclusions we draw from these observations are :—

1. That the rise in the opsonic index following the injection of the antitoxic and antibacterial sera, is, on the whole, comparatively slight.

2. That the depressing effects are more marked.

These effects cannot altogether depend upon the injection into the human body of a foreign serum, as they were much more marked in the case of antitetanic serum than in the other two. The probability is that the injection of the tetanus toxin into a horse, causes it to form some substance which is very toxic to the human body. These toxic effects are indicated by :—

- (a) Several symptoms, *e.g.*, skin rashes, albuminuria, oedema, and a feeling of great debility.
- (b) Lowering of the opsonic index to most of the common organisms.
- (c) By something in the serum which produces an injurious effect on the red blood corpuscles and thus enables the leucocytes to attack and absorb them.

At first sight it is not easy to see why, after the injection of the various sera, there should be a rise in the opsonic index at all. This may perhaps be explained in one of two ways. Firstly, that the various antitoxic antibacterial sera contain opsonins.

In order to find out whether such is the case or not, these sera (antistreptococcal and antidiphtheric) were taken instead of human serum and were incubated for 15 minutes with equal parts of washed leucocytes and an emulsion of the particular organism, *i.e.*, diphtheria and streptococcus. No phagocytosis was observed. This seems to indicate one of two things :—

1. That the various sera, although they must have contained opsonins when freshly prepared (as they are made by injecting horses with what is practically a vaccine) may not after having been kept for several months contain any ; or
2. That human leucocytes will not exert their phagocytic power in the presence of a serum other than human.

However, it was found that washed human leucocytes will take up bacteria in the presence of freshly drawn cat's serum. Using human leucocytes and an emulsion of staphylococci it was ascertained that the opsonic index of cat's serum as compared with human was .6 thus shewing that human corpuscles have a considerable phagocytic action in the presence of cat's serum.

This would seem to show that the various antitoxic and antibacterial sera which, as is usually the case, have been kept for at least several weeks before use, do not contain any opsonins. Therefore the rise in the opsonic index which was noted may be due to either :—

- (a) Substance in the sera which is easily converted by the body into opsonins, or
- (b) As is more likely, to vaccines which are present in the antitoxic and antibacterial sera and which when injected into the body stimulate it to form opsonins.

## A STUDY OF THE INFLUENCE OF NITRITE OF SODIUM ON ANIMAL METABOLISM<sup>1</sup>

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The nitrites, both organic and inorganic, have received considerable attention since the time that Sir Lauder Brunton recommended the use of amyl nitrite for cutting short an attack of angina pectoris on purely physiological grounds. Sodium nitrite has been used for the same purpose, or better still, for preventing the onset of an attack of this disease, and also in asthma, on the supposition that the latter disease is produced by the spasmodic contraction of the smaller bronchioles. Again, recently nitrites have been recommended in haemoptysis, the idea being to produce dilatation of the peripheral blood vessels and thus lessen the amount of blood going to the lungs. However, the present investigation has been undertaken not for the purpose of studying this action of sodium nitrite, but to see whether any change is produced in animal metabolism by subcutaneous use of the drug.

Petrone and Darschkewitsch claim that in syphilis and tabes subcutaneous injection of sodium nitrite in gradually increasing doses produces considerable amelioration of the condition of the patient, the dose varying between 0.01 to 0.06 gram per diem. The favourable result is attributed to the antisyphilitic and antiseptic properties of the drug. It seems too much to expect much antiseptic effect from such small quantities of a comparatively mild antiseptic like sodium nitrite. As for the specific antisyphilitic property, the explanation is stating the result in other words, and does not bring us nearer the mode of action of the drug. It appeared that perhaps a study of

1. For this research a grant was given by the Government of Bombay.

the metabolic changes occurring in the dog under the influence of the drug might show some results which may bring us nearer the truth.

Four such observations have been made, and the last series of observations were continued for a sufficiently long period to show any change that might be produced. The following is the general plan that was adopted in these observations :—

The animal was accustomed to live in a cage specially designed for these experiments, and its weight was observed for a few days before the experiment was started. As soon as the weight remained constant it was kept on a particular diet ; this consisted in all cases of 100 grams of Spratt's dog biscuits powdered and mixed with 100 grams of sterilised minced meat, with a known quantity of distilled water, viz., 200 c.c. The amount of water in the meat was estimated.

The faeces and urine were analysed for water, fat, and nitrogen.

The fat was estimated by Soxhlet's method, while Kjeldahl's method was employed for nitrogen estimation. Urea in urine was estimated with hypobromite of soda.

The weight was observed daily to see whether the animal was in metabolic equilibrium or not. As the animals did not have a motion every day, it is necessary to take the average result of the different periods for the purpose of comparison. The drug used was Merck's purified sodium nitrite sticks suitable for injections. It was dissolved in sterilised distilled water. The dose given was 1 c.c. of 1 per cent. solution for Experiment I, while in the other three experiments it was 1 c.c. of a 3 per cent. solution, and in the second series of injections in the last experiment (IV) it was 2 c.c. of 3 per cent. solution. This last amount is equivalent weight for weight to 9 grains of sodium nitrite given hypodermically to a full grown man. This last series was continued for four days, however no evil effect was noticed in the dog even temporarily.

The principal facts noticed during these experiments were that all the animals slowly increased in weight during the period of

observation, and that none of them went off its feed or became restless.

For the purpose of comparison the daily tables are not satisfactory as, although the animals were thoroughly accustomed to live in the experimental cage, occasionally, for some unknown reasons, a marked daily variation has been produced in the urinary secretion ; however, on taking an average of each period, these variations have become very slight.

Thus the averages of the amount of urine passed in twenty-four hours show a distinct increase in all except in Experiment IV, first series, which shows a diminution. This is probably due to some experimental error, in as far as on the first day of the normal period the animal voided a large amount of urine—perhaps through some oversight the dog had an extra amount of water on the preceding day. On the second and third days the amount fell steadily, and if we take the averages of these two days in conjunction with the quantity of water in the faeces, the result is the same as in the other three experiments, *i.e.*, the total of the urinary secretion and the water in the faeces is much larger during the period of drug administration. Again, if we take the averages of all normal periods as far as the amount of urinary secretion is concerned, and compare them with those of the nitrite period in Experiment IV, we find that the amount of water is increased during the latter. The increase is about 5 per cent., while if the first normal day is excluded the increase is about 8 per cent. during the nitrite period.

Most of the authorities I have been able to refer to are of opinion that nitrites have no marked effect on urinary secretion. The slight increase in the amount of fluid noted by them is said to be due to the dilatation of the blood vessels of the kidneys. None believes that there is any increase of urea or total nitrogen. *Cushny and Sollman*, in their works state that the urine may be increased or diminished according as the dilatation of the blood vessels of the kidneys preponderates over that of the general peripheral vessels or *vice versa*.

In my experiments the increase of water noticed in almost all the cases is not less than 10 per cent and not higher than 32 per cent. ; in

Experiment IV the first series forms an exception, but, as stated above when the first day of the first normal is not counted, the increase is about 8 per cent on the whole experiment.

Excretion of urea and nitrogen is also fairly increased in all the cases without exception ; in Exp. I the animal had a very small dose and the increase is also slight. This at least indicates something more than simple dilatation of the vessels, again these effects are partly kept up during the succeeding normal periods which also indicates something more than mere vaso-dilatation. The increase of urea is neither less than 8 per cent. nor greater than 26 per cent., while the increase of nitrogen varies between 7 and 24 per cent.

As regards the absorption of water from the alimentary canal, Exp. I, II, and III show an increase to the extent of 15 to 20 per cent., on the other hand, the percentage of solids as compared with that of water was also increased in these three experiments. In Exp. IV the last series of observations as compared with the last normal shows the same result. This dog was probably less susceptible to the action of the drug as a larger dose produced results similar to those of the other experiments, besides it is probable that the animal was not in metabolic equilibrium in the commencement of the experiment, thus the amount of urine passed on the first day was considerable as has been stated above.

As regards the question of absorption of nitrogen and fat, Exp. II, III, and IV (with the larger dose) show a distinct increase, while Exp. I and IV (with the smaller dose) show a diminution, this may be due to the extremely small dose in Exp. I, and to the insusceptibility of the last animal. The increase of nitrogen absorption varied between 7 to 14 per cent., while that of fat varied between 9 to 21 per cent.

As regards the explanation of the various effects observed on the urinary secretion and absorption of food stuffs from the alimentary canal we may suppose that the dilated state of the renal vessels might be strong enough to produce the increased flow of water, but it is not quite enough to account for the increase of total nitrogen and urea. This certainly seems to show something more than mere dilatation of the blood vessels. This may be due to the greater excretory action of

the renal epithelium only or to increased functional activity of the liver. To support the latter view we may note that the nitrites produce temporary glycosuria (Cushny and Sollman).

During the period of drug administration we have seen that the amount of faeces was diminished—this may be due to interference with the peristaltic action of the bowels. Vapour of amyl nitrite is said to lessen considerably the activity of unstriated muscles, as in the case of blood vessels, intestines, and ureters (Cushny).

Thus on account of this effect on the muscles of the intestines it is likely that the food remained in the canal for a longer while and thus greater absorption of nitrogen, fat, and water took place as we have found in the experiments.

The dilating effect of this drug on the blood vessels is only temporary even when the drug is given by mouth—and the dose is about 2 grains. Larger doses produce symptoms of collapse and gastric irritation, the latter being due probably to the setting free of nitrous oxide by the action of the HCl of the gastric juice.

In case of hypodermic use, the dilating effect is also temporary and not much perceived even after a dose of 3 grains, while on the other hand the reflex irritability of muscles is distinctly lowered and the effect is fairly permanent, thus exaggerated knee-jerk and ankle-clonus become much less marked.

Again, the hypodermic method is perfectly harmless, no local pain or swelling is noticed, and the organism can stand much larger doses. The average dose of 0.05 to 0.5 gram divided into two portions per day has been found quite safe (*Medical Annual*, 1897, p. 554).

These facts coupled with the results of the experiments mentioned above show that this drug has other properties besides that of producing dilatation of blood vessels. That tissue change and absorption of food stuffs are affected by nitrites is almost certain, as the hypodermic use of nitrites, particularly amyl nitrite, has been found to be followed by glycosuria in some observations (Cushny and Sollmann).

All these facts seem to show that the beneficial influence, if any exists, of the drug in syphilis, and especially nervous affections following syphilis, is probably due to the improved nutrition of the organism.

It is a curious fact observed clinically in my cases that the drug is comparatively less efficient in dilating the blood vessels when injected subcutaneously as compared with its action when taken *per Os*. Thus an injection of 3 grains produces very slight fulness of the blood vessels, while the same dose by mouth produces symptoms of collapse in some cases. Is it not probable that the rapid dilatation produced by this drug when given by mouth may be due to the liberation of nitrous oxide by the gastric juice and the subsequent absorption of the gas? On the other hand, this decomposition cannot take place in the alkaline tissues of the animal organism, and the milder effect is due to the undecomposed drug. It has been stated by Browning (*Therapeutic Gazette*, 1901) that the effect of the drug is temporary when injected subcutaneously as it is rapidly eliminated from the system; but this is not the fact, as its presence can be detected in the urine even after 24 hours in a person who had an injection of 3 grains on the previous day.

My best thanks are due to the Government of Bombay for having given me a grant for this research.

## EXPERIMENT I. SHOWS THE AVERAGES OF EXCRETION OF DOG I

DAYS	URINE			FAECES		
	Quantity c.c.	Total urea grms.	Total nitrogen grms.	Total quantity grms.	Total solids c.c.	Total water c.c.
<i>Averages of four normal days ...</i>						
124	4.94	2.61	4.8	12.99	35.01	27.06
136	5.31	2.81	4.597	15.12	30.85	32.90
<i>" four sodium nitrite days ... (1 c.c. of 1 % solution)</i>						
139	5.97	3.00	4.964	14.93	34.70	30.08
<i>" four days following treatment</i>						

## EXPERIMENT II. SHOWS THE AVERAGES OF EXCRETION OF DOG II

DAYS	URINE			FAECES		
	Quantity c.c.	Total urea grms.	Total nitrogen grms.	Total quantity grms.	Total solids c.c.	Total water c.c.
<i>Averages of three normal days ...</i>						
100	8.38	3.88	53.35	17.10	36.45	31.92
132.5	10.17	4.68	44.35	14.22	30.14	32.06
<i>" three sodium nitrite days ... (1 c.c. of 3 %)</i>						
135	9.60	4.43	33.50	10.45	22.55	32.69
<i>" two days following treatment</i>						

## EXPERIMENT III. SHOWS THE AVERAGES OF EXCRETION OF DOG III

DAYS	URINE			FAECES		
	Quantity c.c.	Total urea grms.	Total nitrogen grms.	Total quantity grms.	Total solids c.c.	Total water c.c.
<i>Averages of three normal days ...</i>						
122	7.95	3.56	39.63	14.33	25.30	36.15
152	8.62	4.02	33.06	12.75	20.31	38.58
<i>" three sodium nitrite days ... (1 c.c. of 3 % solution)</i>						
170	10.47	4.34	38.47	12.24	26.23	33.48
<i>" four days following treatment</i>						

#### EXPERIMENT IV. SHOWS THE AVERAGES OF EXCRETION OF DOG IV

DAYS	URINE			FAECES				
	Quantity c.c.	Total urea grms.	Total nitrogen grms.	Total solids grms.	Total water c.c.	Water %	Total nitrogen grms.	Total fat grms.
Averages of three normal days	...	163	7.70	3.78	28.55	9.95	18.60	0.75
,,   four sodium nitrite days (1 c.c. of 3 % solution)	147	8.38	4.26	41.69	13.12	28.57	31.49	0.67
,,   four days following treatment	150	9.90	4.84	32.28	11.44	20.84	35.43	0.62
,,   four sodium nitrite days (2 c.c. of 3 % solution)	175	10.36	4.94	32.52	11.31	21.21	34.79	0.58
,,   one day following treatment	140	8.72	4.77	40.01	12.55	27.46	31.36	0.73
Normal day	...	110	7.35	3.36	56.92	19.58	37.34	1.17
,,   ,,	...	2	8.0	5.77	27.3	...	...	...
,,   ,,	...	3	175	10.73	4.58	61.97	23.40	1.60
Sodium nitrite day (1 c.c. 3 % sol.)	4	110	7.65	3.53	...	...	...	...
,,   ,,	5	80	9.10	4.61	46.74	20.14	26.60	1.44
,,   ,,	6	65	9.10	3.93	52.44	18.16	34.28	1.05
Normal day following treatment	7	190	10.06	4.11	...	...	...	...
,,   ,,	8	180	10.46	4.38	76.60	26.16	50.44	1.74
,,   ,,	9	150	10.20	4.33	...	...	...	...
,,   ,,	10	160	11.14	4.56	69.30	22.80	46.50	1.38

### EXPERIMENT III. Shows the Daily Excretion of Dog III

Normal day	...	...	1	110	7.35	3.36	5.692	19.58	37.34	34.40	65.60	1.17
"	"	...	2	80	5.77	2.73	...	...	...	...	...	...
"	"	...	3	175	10.73	4.58	61.97	23.40	38.57	37.74	62.26	1.60
Sodium nitrite day (1 c.c. 3 % sol.)			4	110	7.65	3.53	...	...	...	...	...	...
"	"	"	5	180	9.10	4.61	46.74	20.14	26.60	43.10	56.90	1.44
"	"	"	6	165	9.10	3.93	52.44	18.16	34.28	34.60	65.40	1.16
Normal day following treatment			7	190	10.06	4.11	...	...	...	...	...	...
"	"	"	8	180	10.46	4.38	76.60	26.16	50.44	34.15	65.85	1.73
"	"	"	9	150	10.20	4.33	...	...	...	...	...	...
"	"	"	10	160	11.14	4.56	69.30	22.80	46.50	32.90	67.10	1.38

# ON THE USE OF SOLUBLE PRUSSIAN BLUE FOR INVESTIGATING THE REDUCING POWER OF ANIMAL BIOPLASM

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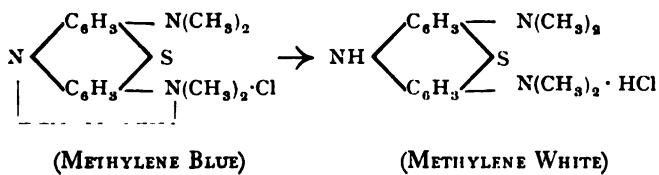
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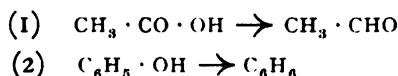
The reducing-power of living animal tissues, of bioplasm, is a property of the first importance from the bio-chemical standpoint. Normally it is exerted upon oxyhaemoglobin, which yields some, not all, of its dissociable oxygen to the living cells through the inter-mediation of the lymph. This is a case of direct de-oxidation : on its efficiency depends the continued existence of the bioplasm.

Living tissue can, however, act as a chemical reducer under circumstances which do not admit of de-oxidation. This is shown in Paul Ehrlich's (1) well-known experiment in which methylene blue injected *intra vitam* into the circulation is reduced to a green or even colourless condition in the more active organs.

Some confusion seems to exist as to the precise nature of this change, as, despite the fact that methylene blue contains no oxygen, the reaction is frequently referred to as one of de-oxidation (2 and 3). In this particular instance the reduction is due, not to the abstraction of oxygen, but to the addition of two atoms of hydrogen; thus—



and when subsequently the leuco compound (methylene white) is oxidised, these two hydrogen atoms are removed in the form of water, and the colour is regenerated. A reduction of this type differs from such reactions as conversion of Acetic Acid into Acetaldehyde, or of Phenol into Benzene.



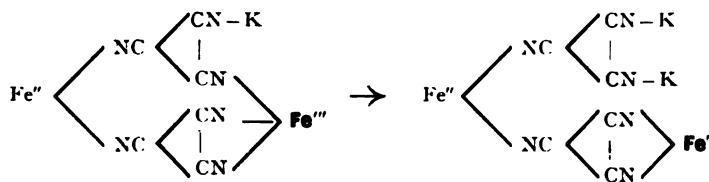
In the former example, the oxygen is *partially* removed by the reducer; in the second case it is *entirely* eliminated. With inorganic substances the mechanism of reduction is frequently complicated, the simplest example being the reduction of a metallic oxide to a metal, but other well-defined types of reducing actions are known, such as the conversion of a Ferric salt to the Ferrous condition.

It will be shown in the present paper that living tissue is capable of effecting reducing actions of this indirect nature, and readily converts soluble Prussian blue to a leuco compound which agrees in its reactions with those of Dipotassio-Ferrous Ferrocyanide.

We consider the reducing-power of bioplasm thus exhibited to be particularly interesting in that not only can living animal matter extract oxygen from such a loose combination as  $\text{HbO}_2$ , and so exhibit the inspiratory phase of the 'internal respiration,' but it can perform such a chemical change as is involved in the transformation of methylene blue to its leuco state (or chromogen), or, as one of us (D.F.H.) (4) pointed out in 1897, the reduction of an appropriate Ferric salt to the Ferrous condition.

As is well known, the *intra vitam* injection of the Prussian blue and gelatine 'mass' often appears from the histological point of view to have been a failure, so pale is the mixture, especially in the capillaries and small vessels. This, we believe, is due to the fact that under such conditions the Potassio-Ferric-Ferrocyanide would be transformed into the Dipotassio-Ferrous-Ferrocyanide; and the reduction of the Ferric to Ferrous condition would be accompanied by a corresponding colour change in which the intense blue of the former compound would become pale green, and ultimately white.

The structural change involved is shown by the following formulae :—



Soluble Prussian blue undergoes the same chemical change when treated with alkaline solutions of organic reducers, *e.g.*, hydroquinone or pyrogallol, so that the tissues may be regarded from the biochemical standpoint as alkaline reducers.

The pale green or colourless compound in the vessels of an injected organ may be restored very rapidly to the vivid blue condition by irrigation with  $H_2O_2$ , just as a piece of Prussian blue gelatine 'bleached' to the leuco state by pyrogallol can be completely 'revived' by the same treatment. This revival can be brought about more slowly by exposure to the air; the colourless condition becoming pale green, the pale green becoming blueish.

In the capillaries of the most active organs—liver and kidney—the reduction from the blue to the colourless condition is so complete that anyone slicing up these organs for the first time invariably says: 'The injection mass has not entered here.' One is often only convinced that it has entered and is still in the organ, on the slices being exposed to the air or irrigated with  $H_2O_2$ , when, as one watches the cut surface, the streaks of injected vessels emerge into visibility like letters developing in 'sympathetic' ink. Seen for the first time, this is very striking—more so, we think, than the corresponding methylene blue revival.

One of the most convincing demonstrations of the reducing power of the tissues as exerted on soluble Prussian blue was carried out in the isolated kidney of the pig which was actually induced to excrete an artificial, gelatinous, colourless 'urine.' A pig's kidney, an hour or two after the death of the animal, was first of all washed out with 75% saline at 42°C., so that all traces of blood were removed, and colourless saline was running from the renal vein and from the

ureter, 6 cm. of which had been preserved. Gelatine and Prussian blue was then substituted for salt solution ; when the pressure of injection approached 210 mm. of mercury we noticed a colourless viscid liquid dropping from the ureter, and, subsequently, on chilling the organ and bisecting it, we found the pelvis and calices of the kidney full of colourless gelatine, portions of which, on irrigation with  $H_2O_2$ , immediately became of a bright blue colour.

It was not until we had thus restored the colour with an oxidiser that we assured ourselves that the colourless excretion contained none other than the Dipotassio-Ferrous-Ferrocyanide, which we were now able to change back to the Potassio-Ferric-Ferrocyanide ; the reduction had been carried by bio-chemical activity to a more perfect leuco state than we have ever seen effected by pure chemical reducers, *e.g.*, by nascent H., hydroquinone or pyrogallol. No doubt, the rapid browning which the last two undergo would effect a partial 'masking' of the colourless condition.

Thus gelatine and some soluble Prussian blue had passed through the epithelium either of the Malpighian corpuscles or of the cortical labyrinth, or both ; and, having been in this manner brought into such intimate contact with the living bioplasm, it was reduced with the maximum of bio-chemical vigour from the intensely blue of the Ferric salt to the perfectly leuco condition of the Ferrous salt—a change which involves the reduction of trivalent iron to the divalent condition.

We perfused kidneys with strong solutions of soluble Prussian blue without gelatine, and obtained results similar to those just described as regards the green condition in the capillaries, but were led to believe that such strong solutions (half saturated) of this salt are rather toxic.

The toxicity of the soluble Prussian blue was well shown in one experiment with a sheep's kidney which was being perfused with the gelatine mixture ; in nine minutes after beginning the perfusion, colourless drops fell from the ureter and continued forming for some minutes longer, but on our maintaining the pressure (250 mm. of Hg) the drops began to be greenish, and finally were quite green.

We take this as indicating that as long as the leuco salt came from the ureter, vital reduction was still being effected by the renal parenchyma, but as the cells become poisoned by the Prussian blue they reduced less perfectly, and so allowed the imperfectly reduced form of the salt to be excreted.

In a subsequent experiment we used lamb's or small sheep's kidneys immersed in a shallow tray of  $\text{NaCl}$  .75% at  $50^\circ \text{C}$ . Having washed out the vessels with  $\text{NaCl}$  .75% (at low pressures of from 40-110 mm.  $\text{Hg}$ ), we injected the gelatine and Prussian blue mixture at first at a pressure of 50 mm.  $\text{Hg}$ . No flow from ureter occurred till the pressure reached 100 mm., when a drop of a viscid, colourless material was seen forming at the cut end of the ureter ; very slowly it fell, and drop after drop slowly formed—all were colourless. After about a quarter of an hour the drops began to appear greenish, showing that the reduction was becoming diminished in vigour.

We finished with a pressure of 300 mm.  $\text{Hg}$ ., maintained for five minutes, during which time the greenish gelatine continued to flow from the ureter : on disconnecting the injection apparatus and clamping the renal artery of the now very turgid kidney, a greenish material continued to drop from the ureter for the next ten minutes. This greenish substance, on treatment with  $\text{H}_2\text{O}_2$ , became blue.

Even if this result is largely due to filtration through the glomeruli, it is not filtration of unaltered soluble Prussian blue, but of the more or less perfectly reduced form of the pigment—the renal cells having acted as the reducers.

The production of this absolutely colourless Ferrous salt disposes of the view that the change in colour undergone by the Prussian blue in the tissues is merely due to 'fading' brought about by the alkalinity of the tissues (5).

It is, of course, true that such 'free' alkali as the Sodium or Potassium Hydroxide will 'bleach' the gelatine Prussian blue from a deep blue to a pale blue, and then to a colourless condition ; and as a precaution against the effect of 'alkalinity' of tissues, acetic acid is added to the injection mass when prepared by the methods of Paul

Mayer and Ravitz (5). As a matter of fact, the mass that we have always used in these experiments is slightly acid to litmus paper.

Now, in the first place, the alkalinity of tissue is not due to the hydrates of Na or K, but to certain alkaline salts, e.g., sodium carbonate, sodium phosphate, and calcium phosphate.

To imitate the alkalinity of tissues, then, one must use such a solution as Ringer's fluid: this, if added warm to a solution of the gelatine Prussian blue, produces in it no change of colour beyond what would be accounted for by dilution.

As Ringer's fluid is neutral to litmus paper, it was necessary to add sodium carbonate to render it distinctly alkaline, and this mixture behaves with regard to the Prussian blue exactly like the neutral solution.

In addition, the following experiment seems to exclude the possibility of the colour change being due merely to the alkalinity of the tissue. A section of an injected kidney to which the blue colour had been restored by means of  $H_2O_2$  was immersed in very dilute caustic soda solution. The blue colour gradually disappeared, and, as subsequent treatment with oxidisers failed to restore it, the Potassio-Ferric-Ferrocyanide had evidently passed into solution. In this way it is shown that when an injected tissue is oxidised by exposure to air or other means the blue colour then formed is due to the regeneration of Prussian blue. As the leuco compound therefore yields Potassio-Ferric-Ferrocyanide by a process of oxidation, it must in turn have been formed by reduction.

But there is another difference between fading due to alkalinity and to bio-chemical reduction, viz., that whereas the first colour change in alkaline fading is to a lighter blue, the first with reduction is to a green colour. We have never seen the blue of the Ferric salt change to the white condition except through a green stage.

The green intermediate stage is a very common one in cases where the tissue is not sufficiently active to effect complete reduction

1. 75 NaCl saturated with calcium phosphate, and 2 c.c. of one per cent. solution of KCl added for each 100 c.c.

to the leuco stage ; and the leuco stage on exposure to the air often goes back no further than the green stage.

The green stage is only obtained *in vitro* with alkalies used alone when they are in concentration far beyond what they are in blood or lymph, but the green stage is always passed through when the blue gelatine is acted on by an alkaline reducer whether *in vitro* or in blood-vessels.

Injected tissues that have been left blue for at least ten years under alcohol can with great rapidity be turned green and then colourless by alkaline pyrogallol or hydroquinone ; and conversely, tissues in a pale green state for ten years can in a few minutes be restored by  $H_2O_2$  to the blue condition.

Although neither Ringer's fluid alone nor its alkaline modification produces any fading of the colour of the blue gelatine, the addition of a trace of pyrogallol at once bleaches the mixture to a pale green tint.

We therefore conclude that the alkaline salts of blood, lymph or tissues are by themselves not the cause of the so-called 'fading' of the Prussian blue injection—the change of colour in the smaller vessels is due to the pigment having been reduced by the surrounding living bioplasm, which has exerted its reducing influence across the lymph-space and across the capillary wall. That the alkalinity of the blood itself is not a cause of the fading of the Prussian blue is interestingly shown as follows :—In those cases where the Prussian blue has been injected into vessels which still contain some blood the mixture is of a dark *green* colour ; and the stirring up of a little melted blue gelatine with freshly-shed blood leads to the production of *greenish* masses distributed throughout the blood ; or, again, blood added to a dilute solution of blue gelatine turns it pale *green*.

Here we have not the fading to a paler blue due to the alkalinity of the blood, but a certain amount of reduction, small but recognisable, on the part of the blood whereby the blue is changed to green. Compared with the tissues, the reduction in and by blood is, as far as our observations go, minimal. Whether this reduction by blood is due to the 'reducing substances' that are known to exist in it, or to

its leucocytes, is for the present of no moment—the change from blue to green is due to reduction and not to 'fading.'

Physical factors alone cannot account for this change of colour: the addition of blue to red would produce a pure purple, a blue purple or a red purple, as the case might be, but not a green; and so similarly would the addition of red to blue: bio-chemical interaction must here play a part.

In large blood-vessels such as the Portal vein, Inferior Vena Cava, &c., from which all blood has been previously washed out, the gelatine mass shows no alteration in colour whatever; that is, vessels not surrounded by living parenchyma but existing virtually as tubes isolated from tissues, are *not* the seats of reduction—they are not the nutritive centres of oxygen-craving cells, whereas those (capillary) vessels which are divided from the living oxygen-craving tissues only by a lymph-space are the seats of the most energetic reduction.

The relative vigour of the reducing-power of the different living animal tissues can be very well shown by the Prussian blue method. We should say that liver and kidney were the most energetic reducers, the pigment in their capillaries always being very pale green or white; less energetic we should call alimentary mucosa, glands, brain and skin; the spleen and lung are either feeble reducers or are easily poisoned. Capsules of organs have little power of reduction compared with the parenchyma they envelope. We are in doubt as to where to place muscular tissue, and hope shortly to make its reducing-power the object of more detailed study.

#### SUMMARY

1. Potassio-Ferric-Ferrocyanide injected into blood-vessels *intra vitam*, and retained in them *post-mortem*, by being transformed to the green or white Di-Potassio-Ferrous-Ferrocyanide, demonstrates the bio-chemical reducing-power which living tissues possess.

2. This change is not due to the alkalinity of the tissues, although that condition provides for reduction in an alkaline medium.

3. The reducing-power of the renal tissue in particular can also be strikingly demonstrated by perfusing the soluble Prussian blue and gelatine through a surviving kidney until a flow from the ureter of the leuco or pale green salt has been established.

4. Whereas filtration under pressure is undoubtedly *the* factor producing this last-mentioned result, there is present in addition the factor of bioplasmic reduction—the chemical basis of the respiratory phase of tissue respiration.

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**A CONTRIBUTION TO THE EXPERIMENTAL PATHOLOGY  
OF CATARRHAL JAUNDICE**

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'The discovery by Bayliss and Starling<sup>1</sup> that the mechanism of pancreatic secretion was of the nature of a chemical reflex has opened up many important lines of research both in physiology and in experimental pathology. Stated in its most simple form, this mechanism consists in the formation in the upper part of the small intestines of a substance called Secretin, which, entering the blood stream and thereby coming eventually into contact with the cells of the pancreas, excites this organ to activity and produces a copious flow of juice. Secretin is only formed when acid is present in the contents of the duodenum and jejunum : the acid is supposed to act upon a 'mother substance' prosecretin in the epithelial cells, and to produce therefrom this secretin. As long as acid is in contact with the mucous membrane in this tract of bowel, so long will secretin be formed ; eventually the alkaline pancreatic flow provoked by the secretin will neutralise the duodenal contents, and thereby further formation of secretin will cease. Later research has demonstrated that secretin is also a stimulant to the liver and intestinal glands.<sup>2</sup>

The acid, therefore, in the stomach not only acts as a necessary factor in peptic digestion, and also a trap for bacteria, but in a lower portion of the alimentary canal it is necessary for the proper stimulation of liver, pancreas, and intestinal glands.

Some important applications of this discovery to pathology have already been given by Starling,<sup>3</sup> but hitherto no attention has been

1. Bayliss and Starling, *Journal of Physiology*, Vol. XXVIII, 325, 1902.
2. Starling, *Croonian Lectures*, 1905.
3. Starling, *Trans. Pathol. Soc. London*, 1903.

given to its application to the experimental pathology of catarrhal jaundice. In catarrhal jaundice the most evident and obtrusive signs and symptoms are those due to stasis and re-absorption of bile. It is singular how little attention is paid to the result of obstruction of the pancreatic duct which occurs in this form of icterus, though the pancreatic juice contains three of the most powerful digestive enzymes, and is also concerned largely with the neutralization of the chyme by means of the alkaline carbonate which it contains.

In health the stomach contents are acid, but in the duodenum there is, as we have seen, an automatic mechanism, namely, secretin formation, which ensures neutrality. Here peptic digestion must obviously come to a standstill, as the acid reaction essential for its activity is no longer maintained. But secretin has not only summoned alkali for neutralization purposes, it has brought also in the pancreatic juice at least four enzymes which will proceed to effect hydrolysis and digestion of the neutralized duodenal chyme.

Suppose now (what actually happens in catarrhal jaundice) that both bile and pancreatic juice are prevented from entering the duodenum. If the stomach still secretes hydrochloric acid, then this acid will pass into tracts of bowel which have hitherto been accustomed to enclose neutral or only faintly acid or alkaline contents. This cannot fail to act in a disturbing manner.

If prosecretin continues to be present in the duodenum and jejunum, then the continued acidity of their contents will produce vigorous formation of secretin, which, if the obstructed organs still retain their sensitiveness to this stimulus, will continue to urge both pancreas and liver to greater activity although no direct outlet for the secretions is available. This increased formation of secretin will, however, have this important advantage, that the flow of succus entericus will be greatly increased, thereby neutralizing the acid jejunal contents and bringing large quantities of the proper ferment of the intestines to bear upon the merely peptonised chyme.

If, however, no acid is produced in the stomach, then no secretin will be formed, and the liver and pancreas will not be stimulated to useless efforts, and the contents of the duodenum and jejunum will

not possess an irritating acidity. Against these advantages there must be noted that peptic digestion will be abolished, that bacteria will have ready entrance, and that the proper stimulus to the intestinal glands will be absent.

The questions which such theoretical considerations raise may be grouped as follows :—

- (1) If pancreatic duct or pancreatic and bile duct together be obstructed, is acid still found in the stomach ?
- (2) If so, does prosecretin continue to be present in the mucous membrane of duodenum and jejunum ?
- (3) Do the pancreas and liver continue to be responsive to secretin when egress for their secretion is denied ?
- (4) Does acid chyme under such circumstances extend for any distance down the small intestine ?

#### CHEMICAL METHODS

Investigation of the stomach contents was confined solely to the relative amounts of hydrochloric acid secreted. No attempt was made to differentiate between free and 'combined' hydrochloric. The term 'combined hydrochloric' seems to cover not only the acid held in loose combination (adsorption or reversible molecular combination), which has still acidic properties—that is, has a certain hydrogen ion concentration—but also the more definite compound obtained by the proteid becoming basic and uniting with the acid to form a stable chloride. As I have been unable to discover any data giving the hydrogen concentration of these compounds, nor of the hydrogen ion concentrations, which are denoted by the end-points of the indicators employed, I have decided not to employ titrations such as those with alizarin and dimethyl-amido-azo-benzol, as they cannot but be empirical.

I have also noted in a dog on which oesophagostomy had been performed, that the animal remained in perfect health (and even laid on flesh) though no free hydrochloric acid was detectable by the Gunzberg reaction in the stomach after a meal rich in proteids. Here practically all the acid was combined with proteids. This fact

supports the contention of many physiologists that acid over and above what is necessary to saturate the proteids present is not an essential factor in gastric digestion. The method employed for estimating the hydrochloric acid was a modification of that of Lüttke, and had better be described in detail :—

A meal of steamed dog biscuit was given to the dog in the morning, and one and a half hours afterwards a sample of the stomach contents was withdrawn and filtered. Two equal portions, 'A' and 'B,' of the filtrate, measured by pipette, were placed each in a porcelain crucible. To 'B' slight excess of chloride-free sodium carbonate was added. Both were placed on a water bath, allowed to evaporate to dryness, and then calcined over a weak bunsen flame until all burning had ceased. The residue in each case was repeatedly extracted with distilled water. In each extract the chlorides present were estimated by the Volhardt method. That is to say, nitric acid in a few drops of iron-alum solution were added to each, and then a measured excess of  $\frac{N}{10}$  silver nitrate.

The total volume of each was diluted with distilled water to the same volume, and one expressible in round numbers, then filtered, and half the filtrate taken and titrated with  $\frac{N}{10}$  ammonium sulphocyanide until a red colour appeared.

The difference in each case between the amount of  $\text{AgNO}_3$  and double the amount of  $\text{AmCNS}$  gives the amount of chlorides present in each calcined residue. The difference finally between the chlorides of each residue represents the amount of hydrochloric acid expressed in c.c. of decinormal strength present in the portion of filtered juice 'A' or 'B.' From this the normality of hydrochloric acid in the filtered juice can be calculated.

*Criticism of the Method.*—It will be seen from the above that this method of analysis aims at determining in a sample of the filtered contents of the stomach the amount of hydrochloric acid which has been added by the gastric glands.

As chlorides of the physiological metals are volatile at temperatures easily reached in calcination wrong results will be given if volatilisation occurs to a greater or less extent in 'A' than in 'B.' This can be obviated by using a low bunsen flame and taking care not to overheat during calcination.

A source of error which could not be easily obviated would arise if the food contained chlorides of organic bases, as these would decompose in heating, and be estimated as hydrochloric acid. In order to ascertain whether these were present as well as to test the method by a general control, an aqueous extract of the food was made, filtered, and analysed by the above method. The result of the analysis was to indicate that hydrochloric acid was present in a concentration equal to  $\frac{N}{500}$  or 0.007 %, a figure which may be taken to be within the limits of experimental error.

We may, therefore, legitimately assume that when an excess of chloride is found in 'B' (the juice to which sodium carbonate has been added) over 'A,' this excess represents total hydrochloric acid, free, combined, or both together.

## OPERATIVE PROCEDURES

*Experiment I.*—On the 15th February, 1905, a short-haired red terrier bitch was operated upon. After being morphinised and chloroformed, the anterior portion of the neck was shaved and sterilised, and an incision of 2 inches made in the mid-line.

In this operation I decided to perform oesophagostomy rather than oesophagotomy in order to be able to draw off the stomach contents<sup>1</sup>:

- (1) Because the saliva would not be so likely to bathe the wound, and so prevent rapid healing.
- (2) Because the operation promised to be less severe on the animal.
- (3) Because it appeared possible that the animal might learn to swallow its food in spite of the opening, and thus would be very much easier kept in a state of health, and approach more nearly to a normal condition.

The oesophagus was exposed and separated from its attachments just below the level of the thyroid cartilage, great care being exercised to avoid cutting the recurrent laryngeal nerve, as it lies on this part of the oesophagus.

The oesophagus being then drawn up into the wound, was opened by a longitudinal incision one inch in length down its centre, and a continuous suture of fine cat-gut was sewn right round the cut edges, so as to obliterate all raw surface.

The oesophagus was now sewn to the skin wound by cat-gut sutures passing through the sterno-mastoid muscles and out through the skin, so holding it in place.

A large opening was thus formed leading into the oesophagus, the suturing completely obliterating all raw surfaces, and the mucous membrane being closely approximated to the skin.

The wound was dressed with boracic acid and cotton wool and a gauze bandage, and it was remarkable with what rapidity healing took place, for in 24 hours the dog was lively and apparently well.

On February 22nd, several meals of bread having been given for a week—one meal each day being left in the stomach for one and a quarter hours—it was found that hydrochloric acid was present by qualitative tests. The dog kept in splendid condition, and except for the test meals of bread was fed on raw meat minced in a sausage machine.

After trying feeding by forcing the minced meat through a syringe for some time, it was found not only very troublesome, but the animal was scarcely getting enough.

Placing a pad of indiarubber over the oesophageal opening was then tried, and it was found that not only could the dog swallow minced meat when taken by the mouth, but it could eat small pieces equally well and lap water. Indeed, it was ultimately proved that the animal got so accustomed to the oesophageal opening that it could soon eat its meals as comfortably in the new condition as before operation. This materially assisted the course of the experiments, and made it much easier to keep the dogs in good health.

1. The operation of oesophagotomy described by Pawlow (*Ergebnisse der Physiologie* I, Abt. i, 246, 1902) had previously been performed on another dog, but the animal lost flesh and the neck wound did not heal well.

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On 23rd February, after a test meal of bread, 20 c.c. of filtered gastric juice was quantitatively analysed for hydrochloric acid.

The amount of this acid stated in terms of normality was  $\frac{N}{15.6}$ .

On 25th February, 50 c.c. showed a concentration of hydrochloric acid equal to  $\frac{N}{27.1}$ .

On 27th February, 40 c.c. showed a concentration of hydrochloric acid equal to  $\frac{N}{30.3}$ .

From this time on till the 22nd May, the dog kept in perfect condition and was used at times for demonstration in class.

On the 22nd May, after being morphinised and anaesthetised by chloroform and ether mixture the abdomen was shaved, cleansed by scrubbing with ethereal soap and hot water, then with turpentine, and the skin made aseptic with biniodide of mercury solution 1-500. An incision 3 inches in length was made in the mid-line between the ensiform cartilage and umbilicus. The stomach and intestines were found to be greatly distended, probably because a sufficiently long interval was not allowed to elapse between the last meal and commencement of operation. This distention rendered the operation very difficult.

The duodenum was lifted and the pancreas exposed. The vessels near the duct were ligatured with silk and cut, and the duct dissected out, tied in two places with silk, and cut across between the ligatures.<sup>1</sup>

The abdominal wound was then closed in layers, as follows:—(a) The peritoneum was closed by a continuous cat-gut suture. (b) Two silk-worm gut sutures were then introduced through skin, muscle, and aponeuroses on the one side and brought out through aponeuroses, muscle, and skin on the other, and left untied in the meantime. (c) Muscle and aponeuroses were brought into apposition by a continuous cat-gut suture. (d) The skin by a continuous cat-gut. (e) The silk-worm gut sutures were then tied.

The wound was dry dressed with boracic powder, a single strip of gauze, and collodion. Dry gauze was placed over this and a wide flannelette bandage applied. The dog never rallied from the operation and died on the third day from shock.

*Experiment II.*—On the 19th August, oesophagostomy was performed on a white fox terrier bitch, the same methods being employed as have already been given in detail. The dog recovered rapidly, and the wound healed by first intention. This dog, when received into the laboratory and when operated upon was in rather poor condition, so that, before making experiments it was decided to feed freely with raw meat in order to allow the animal to put on flesh. This was accordingly done in the manner already mentioned, namely, by holding a pad over the opening in the throat and giving the food, which was readily swallowed by the mouth.

1. The small duct of the pancreas which enters the duodenum near the pylorus was not tied, as it is of such small calibre that its influence upon digestion may be disregarded, whilst its anatomical position renders it very difficult to reach.

On the 7th September a test meal of steamed dog biscuit was given at 10.5, and at 11.35 the contents of the stomach were syphoned off and filtered. There remained 50 c.c. of clear fluid as filtrate. This after analysis showed a concentration of hydrochloric acid equal to  $\frac{N}{25}$ .

On the 9th September 40 c.c. filtrate of gastric contents showed a concentration of hydrochloric acid equal to  $\frac{N}{35.7}$ .

On the 12th September employing the same technique as before described, the abdomen was opened. The stomach and bowels were in this case empty, and the duodenum and pancreas were easily drawn up outside the wound, and the pancreatic duct, after being dissected out, was simply tied with silk so as to occlude its lumen, but was not cut. The abdomen was closed in layers as before, but fine silk was used for the peritoneum instead of cat-gut.

15th September—The dog was very lively, in good condition, and apparently none the worse for the operation. After the usual test meal of steamed biscuit 80 c.c., the filtrate showed a complete absence of hydrochloric acid.

18th September—70 c.c. filtrate showed a concentration of hydrochloric acid equal to  $\frac{N}{75}$ .

20th September—20 c.c. filtrate showed a concentration of hydrochloric acid equal to  $\frac{N}{22}$ .

24th September—70 c.c. filtrate showed a concentration of hydrochloric acid equal to  $\frac{N}{22}$ .

6th November—The dog's health had so far been maintained in apparently perfect condition, but the wound in the neck having cicatrised up was again opened.

9th November—18 c.c. filtrate showed a concentration of hydrochloric acid equal to  $\frac{N}{25}$ .

10th November—30 c.c. filtrate showed a concentration of hydrochloric acid equal to  $\frac{N}{47}$ .

15th November—To find out whether the pancreatic duct was still occluded the abdomen was again opened over the pylorus, an inch to the right of the previous incision. On opening the peritoneum and drawing out the pancreas and duodenum, the duct was found tied with the silk ligatures which had been formerly used. There was a small adhesion of peritoneum and omentum just over the duct. The duct was now ligatured in two places with silk, and cut between the ligatures, when it was seen that it had become patent, showing that simple ligature is not sufficient to interfere with function, as has been noted in ligature of the Fallopian tubes.

18th November—Recovery was complete. 30 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{23.7}$ .

20th November—18 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{75}$ .

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21st November—24 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{27.4}$ .

28th November—The abdomen was again opened in the mid-line on the site of the first incision. Some adhesions were found in the peritoneum, but not of any moment. The peritoneum, however, was rather thin. The dog was well nourished and in excellent condition.

The common bile duct was ligatured in two places with silk at its entrance into the duodenum, and divided between the ties.

29th November—Animal lively, and with good appetite.

2nd December—40 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{20}$ .

3rd December—14 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{18}$ .

4th December—20 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{17.8}$ .

7th December—10 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{15.8}$ .

13th December—20 c.c. gastric juice showed a concentration of hydrochloric acid equal to  $\frac{N}{17.8}$ .

A few days after tying the common bile duct jaundice began to manifest itself by the usual yellow discolouration, which was first evident on the abdomen, spreading thence over the thorax, inside the legs, and up into the neck, becoming deeper in colour as time went on.

As the dog was a white one the icterus showed itself with great plainness, but the general health of the dog appeared in no wise impaired—at least, for the period during which it was under observation—for the animal was as frisky and playful as before, and as eager for its meals.

The faeces were pasty in consistence, and of a slaty colour, and the urine gave the reactions of bile pigment and bile salts in a marked manner.

The dog was fed with meat on the 18th December at 1.30 p.m., and again at 2 p.m. on the 19th, and was killed at 4 p.m. on the 19th.

*Post-mortem Examination.*—The gall bladder and ducts were enormously distended with dark green bile, which was becoming inspissated in the gall bladder. All the ducts within the liver were dilated and filled with fluid, dark green bile, and even sections of the liver gave a green oozing of bile.

The intestinal contents were exceptionally fluid, and the first 2 feet 8 inches of small intestine measured from the pylorus contained food of acid reaction.

The next 3 feet of gut was empty, but the reaction of the moist mucous membrane was acid.

The next 2 feet contained the meal of the 18th instant, and was alkaline.

*Microscopical Sections of Liver and Pancreas.*—With the possible exception of an

increased distinctiveness of the nucleoli when stained with Eosin, sections of the pancreas showed nothing abnormal.

Liver sections also had a normal aspect, the bile ducts having apparently assumed their normal dimensions through elastic retraction.

The next point to determine was whether prosecretin actually existed in the duodenum and jejunum of the animal. If it were absent, then a high degree of acidity could be maintained in the chyme entering the gut and yet the liver and pancreas remain unstimulated, supposing these organs were still responsive to secretin.

The duodenum and jejunum were, therefore, washed and the mucous membrane scraped off with a knife. The mucous scrapings were ground with sand in a mortar, digested with diluted hydrochloric acid, boiled, and whilst boiling made slightly alkaline with potash, and then just acid with acetic acid. On filtering a somewhat opalescent filtrate was obtained, which was now tested for secretin.

A dog was anaesthetised with chloroform, and ether, preceded by morphia injection. Blood pressure was recorded in the usual way from the carotid. The external jugular vein was cannulised for purposes of injection. A cannula filled with water and connected with a rubber tube with a glass nozzle (both filled with water) was next tied into the pancreatic duct.

No flow of pancreatic juice was obtained.

On injection of 5 c.c. of the mucous membrane extract a fall in arterial blood pressure was produced. As the blood pressure began to recover a marked flow of pancreatic juice was observable in the cannula, the drops rising to 10 per minute.

This experiment proves, then, that prosecretin was undoubtedly present in the mucous membrane of duodenum and jejunum, and if this was so, then the acid of the chyme must of necessity have produced secretin, which, entering the blood stream, would eventually reach both pancreas and liver.

We are at this point confronted with two important questions :— First, does secretin, when it enters a pancreas or liver with duct ligatured, excite the organ to further secretory activity ; and second,

is secretin removed from the circulation to a less extent by a pancreas or liver with ligatured duct than by the normal organ?

If the first question be answered in the affirmative, then the greater degree of acidity in the stomach the greater will be the stimulus which the pancreas and liver receive. It must also be noted that the pancreatic juice (and to a less extent the bile) is prevented from reaching the duodenum, the acid of the chyme remains longer unneutralized, and will form secretin for a longer time.

It is difficult to believe that the pancreas or liver continues to be equally susceptible to secretin stimulation after ligature of the duct—in fact, the slow return of colour to the faeces when obstruction causing icterus is removed points pretty clearly to the fact that this response to stimulation has been considerably reduced, and is but slowly regained. Moreover, a recent investigation of this subject by Zunz and Mayer<sup>1</sup> has demonstrated the fact that twelve to fifteen days after ligature of its duct the pancreas could still be excited by secretin, but lost this power later.

If the obstructed organ does not remove secretin from the circulation to the same degree as the normal organ, then more secretin is available for any organ responsive to this hormone and one which is in a normal condition. In the case in point more secretin would be at hand for the stimulation of the intestinal glands even if the concentration of acid in the stomach remained unaltered.

#### DIGESTION IN JAUNDICE

When pancreatic juice is kept back from entering the gut, and thus digestion has to proceed in the absence of trypsin, amyllopsin, and steapsin, it has been found that a compensatory hyperactivity of the intestines is produced<sup>2</sup> which allows the animal to eat and assimilate ordinary food. This compensation must be still more marked when the bile is also prevented from mixing with the intestinal contents, for bile, by reason of its alkalinity, takes some share in

1. *Biochemisches Centralblatt*, Vol. IV, p. 577, 1905.

2. Zunz and Mayer, *Centralblatt f. Physiologie*, Vol. XVIII, 364.

neutralising the acid chyme; hence in its absence after pancreatic obstruction the whole brunt of neutralising the chyme falls on the succus entericus, and until neutralisation is effected secretin will continue to be formed, and the intestinal glands continue to be stimulated.

It is obvious, too, that in such cases peptic digestion will be much more prolonged in duration, for instead of ceasing in the duodenum it will be extended as far down the intestines as the chyme exists unneutralised.

Now, the enzymes present in the intestinal succus are:—

- (a) Erepsin, which transforms the products of peptic digestion, namely, albumoses and peptones, into the same acid products as occurs with digestion with trypsin. Erepsin also acts on the unhydrolysed proteids caseinogen and fibrin.
- (b) Lactase, Maltase, and Invertase, which act on the three disaccharides taken in food and break them down into the monosaccharide sugars, dextrose, levulose and galactose.

Ferments acting on starch or dextrin, fats, and simple proteids, such as albumen and globulin, appear to be unrepresented in the succus entericus, and it is highly improbable that they should be formed *de novo* in the compensatory activity mentioned.

The suggestion has been made that during pancreatic obstruction the ferments formed by that organ are absorbed by the blood and re-excreted in the intestinal juice, but no experimental confirmation of this statement has been advanced. Even if steapsin were thus excreted in the succus the absence of bile salts would prevent absorption of all fatty acids except those like butyric acid, which have a measurable solubility in water. It is much more probable that the digestion of fat which has been detected after ligature of the pancreatic duct was due to bacterial saponification, but in such cases bile was present to dissolve fatty acids of high melting point and low solubility. If bile is absent then, no matter how efficient bacterial hydrolysis may be, only the fats compounded of acids soluble in water can be assimilated.

Whether a diastatic enzyme is present normally in the succus

entericus is a somewhat dubious point ; if it be absent, then if the pancreatic secretion be withheld all starch or dextrin which has escaped digestion in the mouth would remain unabsorbed in the bowel. If, however, it does appear normally or can appear in special circumstances, the compensatory activity of the intestines would surely include this important element in digestion.

One may state, therefore, that when the pancreatic and bile ducts are obstructed normal digestion will still continue to be carried out by the intestine with respect to sugars, proteids which have been changed into albumoses and peptones by the gastric juice, also fibrin and caseinogen even if unaltered by the stomach. The presence of bacteria will confer some slight powers of fat digestion and possibly of starch digestion.

In the light of these facts the increased acidity of the stomach contents noted in the dog operated on would have as consequence—

- (1) An increased excitation of the glands of the small intestine brought about by secretin.
- (2) An increase in the digestive powers of the stomach by which more proteids can be transformed into products upon which the succus entericus can act.
- (3) A possible increase in the extent of the tract in which peptic digestion takes place.<sup>1</sup>

As has been stated, the pancreas and possibly the liver become at length insensitive to secretin stimulation, so that a rise of gastric acidity and a consequent rise in the production of secretin would not injuriously affect them.

#### SUMMARY

1. In the animal operated on (dog) ligation of the pancreatic duct was in one case followed by a temporary cessation of hydrochloric acid secretion in the stomach ; in a second operation this was not observed.

1. It is interesting to note that the Russian investigator, Schegelow (Maly's *Jahresbericht*, Vol. XXXII, 396, 1902), found an increased secretion of gastric juice to be consequent upon ligation of the pancreatic duct.

2. Subsequent obstruction of the bile duct in the same animal was followed by an actual rise in the hydrochloric acid secretion of the stomach.
3. After such double ligation, prosecretin still remained present in the duodenal and jejunal mucous membrane.
4. The contents of the duodenum and jejunum were found to be acid for a distance of 5 feet 8 inches ; beyond this point an alkaline reaction was obtained.
5. Merely ligaturing the pancreatic duct failed to produce a permanent obstruction, though the ligature remained tightly drawn and unabsorbed.

My sincere thanks are due to Professor W. A. Osborne for his kindly help and encouragement during the progress of this work.

## THE ACTION OF CARBON DI-OXIDE ON THE RESPIRATION OF THE GOLD-FISH

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*(Received July 9th, 1906)*

Whilst the action of carbon di-oxide on bioplasm generally is that of a narcotic poison, towards certain nerve cells and particularly those governing respiratory movements in the mammal, it behaves as an irritant. But even with these nerve cells, if the tension of the gas is high enough and its administration continued for a sufficiently long time, a condition of narcosis will finally supervene.

In man, according to Haldane and Priestley,<sup>1</sup> the cells of the respiratory centre are exquisitely sensitive to any increase in the  $\text{CO}_2$  tension of the arterial blood with which they are supplied, an increase of 0.2% of an atmosphere causing a doubling of the lung ventilation. According to these investigators the regulation of the extent of pulmonary ventilation is carried out solely by the stimulating action of carbon di-oxide on the cells of the medullary centre.

The following research was undertaken to find out if possible whether alterations in the  $\text{CO}_2$  tensions of water were followed by changes in the respiration of fishes and whether any such regulatory mechanism as has been described above could be found in these animals.

### METHODS

Our investigations were confined to gold fish, as we could obtain them readily and in sufficient number. We hope to continue the research with other fresh-water teleosteans but the great difficulty

1. *J. Physiol.*, Vol. XXXII, 225, 1905.

we have experienced here in obtaining live unwounded specimens has hitherto prevented us from making the extensive series of observations which we had originally planned.

In order to obtain water containing carbon di-oxide at a definite tension two large flasks containing tap water were boiled half an hour and then placed in cold running water and allowed to cool overnight to room temperature. In the one case  $\text{CO}_2$ -free air, and in the other case pure  $\text{CO}_2$  was bubbled through the water whilst cooling and as long as the experiments were in progress. Since the pressure of  $\text{CO}_2$  in one flask was an atmosphere and in another zero and as the water was from the same source, had been boiled for the same time and had the same temperature in each, it will be obvious that in any mixture of the two the  $\text{CO}_2$  tension will be the same percentage of an atmosphere as the volume percentage of the  $\text{CO}_2$ -saturated water. The mixtures were rapidly made in a large Erlenmeyer flask placed close to the fish tank and the fish transferred from the latter as promptly and as quickly as possible. In some cases the same fish was allowed to remain in the Erlenmeyer whilst continuous additions of the  $\text{CO}_2$  water were made. It was found, however, that habituation to the dissolved gas was acquired and the same fish could not be used for experiments again on that day. With the lower tensions of  $\text{CO}_2$  (4% and less) the same fish could be used again after spending an hour in fresh running water.

In a few experiments a mixture of  $\text{CO}_2$  and air (or air plus oxygen) was made in a gasometer and the mixture bubbled for half an hour through water which was continuously shaken. Analyses of the mixture were made from time to time but it was found that though precautions were taken to have the water of the gasometer with approximately the same tension of  $\text{CO}_2$  as the contained air, a slight fall in the  $\text{CO}_2$  percentage of the latter was always found.

As the barometer remained very close to 760 mm. whilst our experiments were being conducted no allowance for this factor was thought necessary in expressing the tensions of  $\text{CO}_2$  in terms of atmospheric percentages.

IRRITANT ACTION OF CO<sub>2</sub>

When a gold-fish was placed in water saturated with CO<sub>2</sub> and at the same temperature as the tank water, it swam violently about and refused absolutely to respire. Soon narcotic symptoms appeared and the fish eventually lay on its side at the bottom of the vessel. We have often observed a very slight movement of the opercula at this stage but none of the mouth. This refusal to breathe is obviously due to the irritating action of the CO<sub>2</sub> on the mouth and gills and is comparable with closure of the glottis in mammals. With lesser tensions the fish after a preliminary refusal would subsequently commence to respire in a shallow way. The exact limit of respirability we could not determine, for, generally speaking, the higher the CO<sub>2</sub> tension the longer was the pause and, as mentioned above, even in water fully saturated with the gas, faint respiratory movements of the opercula could often be observed. In one experiment where the water had been shaken with air containing 27.6% CO<sub>2</sub> and 59.3% O<sub>2</sub>, respiration commenced abruptly after the lapse of two minutes. A similar pause was obtained when water was used which had been shaken with air containing 45.7% CO<sub>2</sub> and 44.7% O<sub>2</sub>. A number of observations were made on this point and it was found that this respiratory pause could be detected with much lower percentages of CO<sub>2</sub>. The lowest in which it was observable was 1.7% CO<sub>2</sub>, obtained by the mixing method. With percentage saturations of CO<sub>2</sub> lower than this we could not detect the slightest action on the respiratory movements. With higher tensions of CO<sub>2</sub>, though we never observed any indications of hyperpnoea, we noted that the respiration was remarkably regular and was altogether free from those voluntary pauses followed by deep gulps in which the normal gold-fish frequently indulges.

## SURFACE BREATHING

It was noted by Duncan and Hoppe-Seyler<sup>1</sup> that when the carp was placed in water with a very low oxygen tension the fish rose and respired the water as close to the actual surface as possible. This

1. *Zeit. f. physiol. Chemie*, Vol. XVII, 165, 1893.

was observed with *tinca vulgaris* when the oxygen tension was 1.84, 0.78 and 0.38% of an atmosphere. The *cobitis fossilis* under similar circumstances rose to the surface and swallowed air. The trout apparently never resorted to this manoeuvre but when subjected to low tensions of oxygen flung itself wildly about, even jumped into the air and showed marked hyperpnoea. This effect of low oxygen tension we have also observed with the gold fish but we believe that the frequent rising of this fish to the surface in order to respire the upper layers, an action which is always seen when the water in which the fish is kept is not renewed sufficiently often, is due to another cause, namely, a rise in the tension of  $\text{CO}_2$ . We have noticed this phenomenon when the  $\text{CO}_2$  tension of the water in which the fish was swimming was raised by a single addition of  $\text{CO}_2$  water from

zero to 3.2% atmosphere  
 zero to 10.7% atmosphere  
 zero to 15% atmosphere  
 3.2% to 6% atmosphere

A fresh investigation was then carried out in which different specimens of the gold-fish were used and in which the  $\text{CO}_2$  tension was raised from zero by small increments. To 3½ litres of  $\text{CO}_2$ -free water small amounts of water (20 to 50 c.cs.) saturated with  $\text{CO}_2$  were added and a couple of minutes allowed for observation. A slight error in calculating the tension was caused by the presence of the fish which, of course, was constantly excreting  $\text{CO}_2$ , but the comparatively large volume of the water and the low metabolism of the fish must have made this error a very small one. The Erlenmeyer flask gave so small a surface that  $\text{CO}_2$  loss during the experiment may also be neglected.

Experiment I	surface breathing occurred at 4% $\text{CO}_2$		
Experiment II	"	"	4% $\text{CO}_2$
Experiment III	"	"	3.8% $\text{CO}_2$
Experiment IV	"	"	4% $\text{CO}_2$
Experiment V	"	"	3.2% $\text{CO}_2$
Experiment VI	"	"	3.3% $\text{CO}_2$

A very interesting feature of this reaction to  $\text{CO}_2$  was the fact that on raising the tension still higher the fish abandoned surface breathing but sometimes resumed it when the tension had reached such figures as 13.9%, 14%, 15% or still higher percentages. There seems then, for the gold fish at least, a critical pressure of  $\text{CO}_2$  which will induce surface breathing and which, according to our experiments, lies between 3.2 and 4% of an atmosphere. Evidence for a second and higher critical point was not so uniform and conclusive.

That this rise to the surface was due to reflex action or instinct and not because the fish consciously swam in the direction of lessened  $\text{CO}_2$  pressure was shown by the speed of the rise and by the fact that it occurred in some test experiments when the atmosphere above the water contains  $\text{CO}_2$  at the same pressure as the water. That it was not due in these cases to oxygen deficiency we were able to disprove completely, for the reaction occurred when the fish was placed in water which had been shaken with air containing not only  $\text{CO}_2$  but high percentages of oxygen (44.7%, 59.3%, 63.9%, 66.3%).

The teleological significance of this response to  $\text{CO}_2$  is obvious. If the  $\text{CO}_2$  tension of the water be raised, either by the fish itself, if the amount and surface of the water be small, or by the evolution of  $\text{CO}_2$  from decomposing organic matter, the fish can still obtain water to respire with a low  $\text{CO}_2$  tension by means of this simple artifice.

#### NARCOTIC ACTION OF $\text{CO}_2$

At what pressure in the water carbon di-oxide begins to exercise a narcotic influence we could not determine owing to the irregular movements of the fish; but with tensions above 10% of an atmosphere the narcosis was marked. At first the fish lay at the bottom of the vessel respiration very regularly but refusing to swim about, though it could still do this in a normal manner if an attempt were made to catch it with the hand. Another sign of narcosis which we found to precede loss of equilibration was the loss of the

head-to-stream reflex. If the water in the vessel were made to rotate in a mass about a vertical axis in the centre, the normal fish at once placed itself in a direction facing the current and by means of swimming movements maintained its position relative to the wall of the vessel and objects visible outside. But the fish poisoned by  $\text{CO}_2$  drifted with the current and with the long axis of its body at any angle to the direction of drift. A sign of more advanced narcosis was the loss of equilibration which was shown by the fish being no longer able to keep its dorsum uppermost. The body at first swayed from one side to another then got a permanent list to one side and finally heeled over completely. Recovery could always be rapidly effected if the fish were placed in fresh running water.

#### SUMMARY

1. Tensions of  $\text{CO}_2$  greater than 1.7% of an atmosphere produce in the gold-fish temporary inhibition of respiration comparable with the closure of the glottis in mammals.
2. Surface breathing can be produced in the gold-fish not only by oxygen deficiency (confirmatory of Duncan and Hoppe-Seyler) but also by  $\text{CO}_2$  at a tension which in our experiments varied between 3.2 and 4% of an atmosphere.
3. The sequence of narcotic manifestations in the gold-fish produced by  $\text{CO}_2$  is
  1. Cessation of voluntary movement except when irritated.
  2. Loss of head-to-stream reflex.
  3. Loss of equilibration.
4. No action of  $\text{CO}_2$  on the respiratory centres comparable with that present in man could be discovered in the gold-fish.

## A NOTE ON THE OSMOTIC EFFECT OF VARIOUS STRENGTHS OF DIFFERENT SALTS UPON CELL VOLUME

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Recently, in a paper by Roaf and Whitley,<sup>1</sup> it was pointed out that tadpoles, when placed in solutions of neutral salts, shrank markedly in size but remained active in spite of this great diminution in bulk. As the shrinkage occurred in solutions which were below the isosmotic equivalent for the blood of full grown frogs, it was surmised that the tadpoles maintain a difference of osmotic pressure between their internal fluids and the surrounding medium. Thus the normal concentration of salts is maintained whilst the organism lives in a solution, such as a fresh water pond, of much lower concentration but if it is placed in a stronger solution of salt, it shrinks until there is a fresh equilibrium established between the two and as the isosmotic strength is approached the degree of shrinkage rapidly increases.

The following experiments were performed to obtain more detailed information regarding these changes. The tadpoles were placed in the solutions in flat earthenware dishes capable of holding about 400 c.c. After being laid on blotting paper, to drain off the superfluous water of the stock tank, they were transferred to a tared watch glass and weighed, they were then put in the solution to be tested. At the end of the experiment they were treated in an exactly similar manner and any loss of weight was calculated as a percentage loss of the original weight.

In several of the experiments the weight of the dried solids was obtained, after rinsing in distilled water and drying at 120° C., then the residue was incinerated and the weight of the ash obtained. This was dissolved and titrated with silver nitrate after Mohr's method.

1. *Bio-Chemical Journal*, Vol. I, p. 88, 1906.

## EXPERIMENT I

Twenty tadpoles (*Rana temporaria*) were placed in 300 c.c. of each of the following solutions and left for 48 hours.

Solution	Original Weight of Tadpoles	Final Weight of Tadpoles	Loss in Weight of Tadpoles	Percentage of Loss to Original Weight
Distilled water	1.646 g	1.363 g	0.283 g	17.19
Tap water	1.590 g	1.314 g	0.276 g	17.36
Tap water + $\frac{M}{100}$ NaCl	1.593 g	1.362 g	0.231 g	14.49
Tap water + $\frac{M}{50}$ NaCl	1.722 g	1.613 g	0.109 g	6.33
Tap water + $\frac{M}{40}$ NaCl	1.740 g	1.659 g	0.081 g	4.59
Tap water + $\frac{M}{20}$ NaCl	1.637 g	1.558 g	0.079 g	4.83
Tap water + $\frac{M}{10}$ NaCl	1.731 g	1.561 g	0.170 g	9.82
Tap water + $\frac{M}{8}$ NaCl	1.826 g	1.318 g	0.508 g	27.82

In  $\frac{M}{10}$  NaCl, one tadpole was found dead, whilst in  $\frac{M}{8}$  NaCl five were found dead, and it is interesting to note the great increase in percentage loss of weight occurring in the strength which kills the tadpoles.

## EXPERIMENT II

Eight tadpoles were placed in 300 c.c. of each of the following solutions and left for 24 hours.

Solution	Original Weight of Tadpoles	Final Weight of Tadpoles	Loss in Weight of Tadpoles	Percentage of Loss to Original Weight
Distilled water	2.382	1.945	0.337	14.1
Tap water	1.952	1.738	0.214	10.9
Tap water + $\frac{M}{100}$ NaCl	2.505	2.374	0.133	5.38
Tap water + $\frac{M}{20}$ NaCl	2.142	2.033	0.109	5.09
Tap water + $\frac{M}{10}$ NaCl	2.116	1.788	0.328	15.5
Tap water + $\frac{M}{8}$ NaCl	1.944	1.669	0.275	14.2 <sup>1</sup>
Tap water + $\frac{M}{30}$ CaCl <sub>2</sub>	2.137	1.734	0.403	18.9
Tap water + $\frac{M}{15}$ CaCl <sub>2</sub>	2.186	1.596	0.596	27.3 <sup>2</sup>
Tap water + $\frac{M}{11.5}$ CaCl <sub>2</sub>	2.445	1.849	0.596	25.2 <sup>3</sup>
Tap water + $\frac{M}{100}$ NaHCO <sub>3</sub>	2.106	1.839	0.267	12.7 <sup>3</sup>
Tap water + $\frac{M}{20}$ NaHCO <sub>3</sub>	2.293	1.864	0.429	18.7 <sup>2</sup>
Tap water + $\frac{M}{10}$ NaHCO <sub>3</sub>	1.851	1.452	0.399	21.5 <sup>4</sup>
Tap water + $\frac{M}{100}$ CH <sub>3</sub> COONa	1.946	1.834	0.102	5.24
Tap water + $\frac{M}{20}$ CH <sub>3</sub> COONa	2.052	1.812	0.240	11.7
Tap water + $\frac{M}{10}$ CH <sub>3</sub> COONa	2.197	1.887	0.310	14.1

1. One dead. 2. All dead. 3. Three dead, several others lethargic. 4. Six dead, one lethargic.

As in the preceding table it is interesting to note the great increase in the percentage loss of weight occurring in solutions which kill the tadpoles.

## EXPERIMENT III

Twelve tadpoles were placed in 300 c.c. of each of the following solutions and left for *four* hours.

Solution	Original Weight of Tadpoles	Final Weight of Tadpoles	Loss in Weight of Tadpoles	Percentage of Loss to Original Weight
Distilled water	1.241	1.142	0.099	8.67
Tap water	1.480	1.387	0.093	6.29
Tap water + $\frac{M}{20}$ NaCl	1.236	1.117	0.119	9.63
Tap water + $\frac{M}{10}$ NaCl	1.175	1.013	0.162	13.79
Tap water + $\frac{M}{8}$ NaCl	1.204	1.003	0.201	16.69
Tap water + $\frac{M}{60}$ CaCl <sub>2</sub>	1.220	1.105	0.115	9.43
Tap water + $\frac{M}{30}$ CaCl <sub>2</sub>	1.048	0.938	0.110	10.51
Tap water + $\frac{M}{20}$ CaCl <sub>2</sub>	1.067	0.940	0.127	11.90
Tap water + $\frac{M}{100}$ NaHCO <sub>3</sub>	1.193	1.133	0.060	5.03
Tap water + $\frac{M}{20}$ NaHCO <sub>3</sub>	1.200	1.110	0.090	7.50
Tap water + $\frac{M}{10}$ NaHCO <sub>3</sub>	1.231	1.086	0.145	10.97
Tap water + $\frac{M}{100}$ CH <sub>3</sub> COONa	1.098	1.011	0.087	7.92
Tap water + $\frac{M}{20}$ CH <sub>3</sub> COONa	1.380	1.262	0.118	8.55
Tap water + $\frac{M}{10}$ CH <sub>3</sub> COONa	1.233	1.059	0.174	14.11

## EXPERIMENT IV

Twelve tadpoles were placed in 300 c.c. of each of the following solutions and left for *four* hours.

Solution	Original Weight of Tadpoles	Final Weight of Tadpoles	Loss in Weight of Tadpoles	Percentage of Loss to Original Weight
Distilled water	1.195	1.103	0.092	7.70
Tap water	1.166	1.115	0.051	4.37
Tap water + $\frac{M}{100}$ NaCl	1.114	1.072	0.042	3.77
Tap water + $\frac{M}{20}$ NaCl	1.051	0.992	0.059	5.61
Tap water + $\frac{M}{10}$ NaCl	1.250	1.153	0.097	7.76
Tap water + $\frac{M}{8}$ NaCl	1.193	1.056	0.137	11.48
Tap water + $\frac{M}{60}$ CaCl <sub>2</sub>	1.136	1.087	0.049	4.31
Tap water + $\frac{M}{30}$ CaCl <sub>2</sub>	1.309	1.239	0.070	5.35
Tap water + $\frac{M}{20}$ CaCl <sub>2</sub>	0.983	0.913	0.070	7.12
Tap water + $\frac{M}{100}$ NaHCO <sub>3</sub>	1.142	1.118	0.024	2.10
Tap water + $\frac{M}{20}$ NaHCO <sub>3</sub>	1.012	0.929	0.083	8.20
Tap water + $\frac{M}{10}$ NaHCO <sub>3</sub>	0.980	0.863	0.117	11.94
Tap water + $\frac{M}{100}$ CH <sub>3</sub> COONa	1.090	1.062	0.028	2.57
Tap water + $\frac{M}{20}$ CH <sub>3</sub> COONa	0.993	0.935	0.058	5.84
Tap water + $\frac{M}{10}$ CH <sub>3</sub> COONa	1.082	0.975	0.107	9.89
Tap water (kept running)	1.168	1.116	0.052	4.45

*Note to Tables.*—There is a large percentage loss of weight which occurs in the tadpoles kept in tap water. This must be due to some condition of experiment, as it is inconceivable that they could continue losing weight all the time that they were kept in the laboratory. In the last experiment a second control was kept in running water to test if that would make any difference, but the loss was the same as with those kept in still water.

The results of these experiments show that there is a certain strength of solution in which the tadpoles are best suited to maintain an equilibrium between their body fluids and the surrounding medium. A decrease in the concentration of the salt causes an increased loss of weight, which reaches a maximum in distilled water. Similarly, increase in the concentration of the salt causes a progressive increase in the loss of weight which becomes more marked as the strength at which the organisms are killed is approached. The great loss in weight which occurs in distilled water is probably due to loss of salts and water from the organism by means of dialysis, aided by an effort on the part of the organism to keep its body fluids of constant concentration. The first effect would be a loss of salt by dialysis and this would be followed by excretion of water in an attempt to keep a constant ratio of salts to water in the animal's body fluids. Addition of a salt to tap water lessens the loss by dialysis and consequently less water would be excreted to keep a balance between the concentration of the fluids of the body and the surrounding medium. At a certain strength of solution the point is reached at which equilibrium is nearly attained between the two solutions but as there is only a single salt in solution the true equilibrium is not found in these experiments. If a mixture of salts were used it is conceivable that at a definite concentration the only loss of weight would be that due to the excretions of the organism during the time of the experiment.<sup>1</sup>

Beyond this optimum strength the loss in strength increases due to loss of water by the increase of osmotic pressure in the surrounding medium. Whether any appreciable exchange of salts occurs could not be determined as the total amount of salt contained in the organism is too small to show any relative change with accuracy but there was, as a rule, a progressive increase in the chlorides in the case where chlorides were present in the external medium and a decrease of chlorides in the case of solutions of sodium bicarbonate and sodium acetate. The dried solids and the ash showed a progressive

1. Cf. Ostwald Wolfgang, *Arch. f. d. ges. Physiol.*, Vol. CVI, p. 568, 1905.

increase as the strength of solution increased, no matter what was the nature of the salt used. It is probably this change in the salts which causes death to occur in solutions of a single salt in distilled water,<sup>1</sup> and when single salts are increased markedly above the other salts (as in using high concentrations of salts in tap water). This latter case has been discussed in a previous paper where the relationship between the proteids and electrolytes has been pointed out.<sup>2</sup>

In view of these facts the current view, that when plasmolysis occurs the solution used is isosmotic with the cell contents, needs to be carefully revised. If the cells used are accustomed to live in fresh water it is extremely probable that they habitually maintain a higher concentration of salt than is present in their surroundings. Any increase in the osmotic strength of the surrounding medium would tend to abstract water but it is only when this pressure is sufficient to overcome the adhesion of the protoplasm to the cell wall that plasmolysis becomes visible.

It is evident from the tables that long before the isosmotic strength is reached there is a progressive loss of weight, as the strength of solution increases; hence showing that it is not equality of pressure but a difference which is maintained by the cell.<sup>3</sup>

In conclusion, I wish to thank Professor Benjamin Moore for his assistance and advice during the course of this research.

1. Loeb and Gies, *Arch. f. d. ges. Physiol.*, Vol. XCIII, p. 246, 1903; also Loeb, *Arch. f. d. ges. Physiol.*, Vol. CVII, p. 252, 1905.

2. Roaf and Whitley, *loc. cit.*, pp. 97-101.

3. Cf. Macallum, *Journ. Physiol.*, Vol. XXIX, p. 213, 1903.

**A CONTRIBUTION TO THE STUDY OF THE DIGESTIVE  
GLAND IN MOLLUSCA AND DECAPOD CRUSTACEA**

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Since the time of Krukenberg many observations have been made on the digestive processes in lower animals. The literature of the subject is extensive but for the purposes of the present paper it is only necessary to mention some of the papers more directly concerned with the experiments described herein. Krukenberg<sup>1</sup> stated that the digestive gland of the crab contained both tryptic and peptic ferments. His reason for stating this was that the tryptic enzyme could be destroyed by the peptic enzyme in acid solution and likewise the peptic enzyme was destroyed by digestion in two per cent. soda solution at 38°—40° C. Frenzel<sup>2</sup> said that a glycerine extract of the gland acted on fibrin like trypsin. Hoppe-Seyler<sup>3</sup> found proteolytic, amylolytic and lipolytic ferments in the digestive gland of the crab. He considered the proteolytic ferment to resemble or to be identical with ordinary trypsin. Jordan<sup>4</sup> claims that the proteolytic ferment of the fresh water crayfish (*Astacus fluviatilis*) is similar to trypsin and that lipolytic and amylolytic ferments are also present.

Frédéricq,<sup>5</sup> in two species of molluscs, found a ferment which dissolved fibrin best in acid reaction whilst in other molluscs the ferment acted best in alkaline media. He also found a diastatic

1. *Untersuchungen a. d. physiol. Institut, Heidelberg*, Vol. II, 1879.
2. *Mittheil. a. d. zoolog. Stat. Neapel*, p. 50, 1883.
3. *Arch. f. d. ges. Physiol.*, Vol. XIV, p. 395, 1876.
4. *Arch. f. d. ges. Physiol.*, Vol. CI, p. 263, 1904.
5. *Bulletins de l'acad. roy. de Belgique*, 2 ser., Vol. XLIV, 1878.

ferment in most of the animals. Griffiths<sup>1</sup> stated that the 'liver' of *Patella vulgata* contains proteolytic, amylolytic, and lipolytic ferment, and MacMunn<sup>2</sup> states that in *Aplysia* there are peptic, tryptic, and amylolytic ferment.

The following species have been investigated during the course of these experiments:—Crustacea: the Edible Crab (*Cancer pagurus*) and the Swimming Crab (*Portunus puber*); Mollusca: the Limpet (*Patella vulgata*), the Periwinkle (*Littorina littorea*), the Dog-whelk (*Purpura lapillus*), and the Hard-whelk (*Fusus antiquus*).

#### EXPERIMENTAL METHODS

The digestive gland was minced, weighed, and mixed with a definite volume of distilled water to which a suitable preservative had been added. After allowing to stand for some time it was strained through muslin and a series of solutions were made up using a definite quantity of the extract. The digestive action was tested for the various ferment by the following methods.

Proteid digestion was sought for by using coagulated egg white (Mett's tubes), fibrin and peptone solution. In no case was there any action on the egg white, but fibrin was acted on in most cases. The degree of action on fibrin was shown by loss of weight and by tests for the products of digestion. Three tests were employed for digested products. (1) Albumoses or peptone (biuret test), (2) tyrosin (testing filtrate from addition of Millon's reagent), (3) tryptophane (addition of either bromine or chlorine water). The effect of reaction was shown by using mixtures which were either acid, alkaline or neutral. For the neutral solutions distilled water was employed. The acid solution, which resulted from mixing a definite quantity of standard acid with the extract of the gland, was 0·18% hydrochloric acid and the corresponding alkaline solution was 0·2% sodium carbonate. The peptone solution was employed in the solutions of varying reaction, to test for eretic ferment, and the same three

1. *Proc. Roy. Soc.*, Vol. XLII, p. 392, 1887.

2. *Royal Soc. Phil. Trans.*, B., Vol. CXCV, p. 1, 1900.

tests were used only in this case the diminution of the biuret pink showed increased digestion, whilst with fibrin the more active the digestion the greater the pink colouration. Carbohydrate digestion was shown by hydrolysis of starch solution and inversion of cane sugar. The tests employed were the non-appearance of the blue colour on the addition of iodine to the starch solution and the presence of a reducing sugar by Trommer's test.

Fat digestion was at first tested by an emulsion of olive oil in most cases, but as the results were uniformly negative, methyl acetate was used in the later experiments and the titration figure with decinormal sodium hydrate in presence of phenol-phthalëin was compared with a control. This latter method gave a marked hydrolysis in every case in which it was used.

As a preservative toluol was employed at first but later on it was found that proteid digestion, as pointed out by Vines,<sup>1</sup> proceeded better when hydrocyanic acid was used. When testing for reducing sugar hydrocyanic acid cannot be used as it decolourizes the copper solution and holds cuprous oxide in solution. A number of experiments were performed and the results were used to check one another, thus individual variations were excluded and a good average obtained.

## RESULTS

Amongst the crustacea it was found that digestion of fibrin proceeded most actively in alkaline reaction, and least in acid solution, neutral mixtures being intermediate in their rate of action. Starch was converted into reducing sugar and cane sugar was inverted in practically every case. Although no hydrolysis of olive oil was found, methyl acetate was readily split into methyl alcohol and acetic acid.

There was no increase in the action on fibrin when intestinal extract was added to the extract of the gland, thus there is no evidence of a substance like enterokinase which can convert an inactive proferment into an active ferment. It is, however, difficult to exclude contamination, and the least trace of enterokinase can

1. *Annals of Botany*, Vol. XVII, p. 597, 1903.

activate a large quantity of inactive juice. Both peptolysis and proteolysis were found to occur and both were most active in alkaline media. Lastly, the proteolytic ferment was destroyed by acid but not by alkali, but the presence of fibrin protects the ferment from the injurious action of the acid, so that some digestion can occur in acid media if the fibrin is added at the same time as the acid.

All the crustacea used gave practically identical results so there is no need for details of the individual species. The following protocols will serve as an example of the method in which an experiment was performed.

May 16th, 2.30 p.m.—57 g Hepato-pancreas of *Cancer pagurus* + 2 c.c. of 2% HCN + 55 c.c. distilled water (added to each and kept at 13° C.)

May 17th, 3 p.m.—The following solutions

were made up and two drops 2% HCN added :—

1. 5 c.c.  $\frac{N}{10}$  HCl + 3 c.c. distilled water + 2 c.c. extract + 0.5 g fibrin.
2. 5 c.c.  $\frac{N}{10}$   $Na_2CO_3$  + 3 c.c. distilled water + 2 c.c. extract + 0.5 g fibrin.
3. 8 c.c. distilled water + 2 c.c. extract + 0.5 g fibrin.
4. 5 c.c.  $\frac{N}{10}$  HCl + 3 c.c., 2% peptone solution + 2 c.c. extract.
5. 5 c.c.  $\frac{N}{10}$   $Na_2CO_3$  + 3 c.c., 2% peptone solution + 2 c.c. extract.
6. 5 c.c. distilled water + 3 c.c., 2% peptone solution + 2 c.c. extract.
7. 5 c.c., 1% starch solution + 3 c.c. distilled water + 2 c.c. extract.
8. 5 c.c., 1% cane sugar solution + 3 c.c. distilled water + 2 c.c. extract.
9. 8 c.c., 1% methyl acetate solution + 2 c.c. extract.

May 18th, 10.30 a.m.—

Swollen and gelatinous, all strains through muslin ; fair biuret, marked tyrosin, no tryptophane.

All digested ; marked biuret, fair tyrosin, slight tryptophane.

0.03 g fibrin left; strong biuret and tyrosin, slight tryptophane.

Good biuret, strong tyrosin, fair tryptophane.

Good biuret, very strong tyrosin, strong tryptophane.

Fair biuret, strong tyrosin, medium tryptophane.

Blue colour with iodine gone, decolourization of Trommer's test.

Trommer's solution decolourized.

With phenol-phthalein = 3.2 c.c.  $\frac{N}{10}$   $NaOH$ .

10. 8 c.c., 1% methyl acetate solution + 2 c.c. boiled extract.	With phenol-phthalein = 1.4 c.c. $\frac{N}{10}$ NaOH.
11. 5 c.c. $\frac{N}{10}$ HCl + 2 c.c. distilled water + 2 c.c. extract after standing 3 hours, 1 c.c. N $\text{Na}_2\text{CO}_3$ and 0.5 g fibrin added.	0.36 g fibrin left; slight biuret, fair tyrosin, very slight tryptophane.
12. 5 c.c. $\frac{N}{10}$ $\text{Na}_2\text{CO}_3$ + 2 c.c. distilled water + 2 c.c. extract, after standing 3 hours 1 c.c. NHCl and 0.5 g fibrin added.	0.13 g fibrin left; strong biuret and tyrosin, slight tryptophane.
13. 5 c.c. $\frac{N}{10}$ HCl + 2.5 c.c. distilled water + 2 c.c. extract, after standing 3 hours 0.5 c.c. N $\text{Na}_2\text{CO}_3$ and 0.5 g fibrin added.	0.38 g fibrin left; fair biuret, medium tyro- sin, no tryptophane.
14. 5 c.c. $\frac{N}{10}$ $\text{Na}_2\text{CO}_3$ + 2.5 c.c. distilled water + 2 c.c. extract, after standing 3 hours 0.5 c.c. N HCl and 0.5 g fibrin added.	All fibrin digested, very strong biuret, strong tyrosin, medium tryptophane.

The extract itself gave strong biuret, tryptophane and tyrosin reactions evidently by auto-digestion, but these effects were negligible when the solution was diluted in the proportions used in the experiments. The digestion of starch and cane sugar are invalidated by the presence of hydrocyanic acid, but similar experiments when toluol was used as a preservative showed hydrolysis of starch and inversion of cane sugar.

The last four solutions (11-14) are very interesting as showing that the ferment is destroyed by acid and is thus rendered incapable of acting on fibrin when rendered alkaline (11) or neutral (13); it is not destroyed by alkali and can act on fibrin after being rendered neutral (14), but not after being rendered acid (12).

With the mollusca the results were very much the same, the main difference being that the digestion of fibrin proceeded most actively in acid media and was practically absent in alkaline reaction. Starch was hydrolysed and cane sugar inverted as in the crustacea. With *Fusus* the 'stomach' was carefully separated from the 'liver' and an extract of it was found to digest fibrin markedly in acid medium, slightly in neutral, but not at all in alkaline reaction, thus corresponding with the results obtained by the extract of the digestive gland.

The following experiment shows these points :—

May 14th, 5 p.m.—35 g Liver of *Fusus antiquus* + 1 c.c. 2% HCN + 34 c.c. distilled water (and kept at 13° C.) Extract of stomach also made.

May 15th, 10.30 a.m.—Following solutions made up, and two drops 2% HCN added to each :—

1. 5 c.c.  $\frac{N}{10}$  HCl + 3 c.c. distilled water + 2 c.c. liver extract + 0.5 g fibrin.
2. 5 c.c.  $\frac{N}{10}$   $\text{Na}_2\text{CO}_3$  + 3 c.c. distilled water + 2 c.c. liver extract + 0.5 g fibrin.
3. 8 c.c. distilled water + 2 c.c. liver extract + 0.5 g fibrin.
4. 5 c.c.  $\frac{N}{10}$  HCl + 3 c.c., 2% peptone solution + 2 c.c. liver extract.
5. 5 c.c.  $\frac{N}{10}$   $\text{Na}_2\text{CO}_3$  + 3 c.c., 2% peptone solution + 2 c.c. liver extract.
6. 5 c.c. distilled water + 3 c.c., 2% peptone solution + 2 c.c. liver extract.
7. 8 c.c., 1% methyl acetate solution + 2 c.c. liver extract.
8. 8 c.c., 1% methyl acetate solution + 2 c.c. distilled water (HCN as others).
9. 5 c.c.  $\frac{N}{10}$  HCl + 3 c.c. distilled water + 2 c.c. stomach extract + 0.5 g fibrin.
10. 5 c.c.  $\frac{N}{10}$   $\text{Na}_2\text{CO}_3$  + 3 c.c. distilled water + 2 c.c. stomach extract + 0.5 g fibrin.
11. 8 c.c. distilled water + 2 c.c. stomach extract + 0.5 g fibrin.

May 17th, 10 a.m.—

- 0.2 g fibrin; very strong biuret, tyrosin and tryptophane.
- 0.47 g fibrin; strong biuret, fair tyrosin, no tryptophane.
- 0.35 g fibrin; strong biuret, marked tyrosin, no tryptophane.
- Very strong biuret and tyrosin, medium tryptophane.
- Very strong biuret, very slight tyrosin, no tryptophane.
- Fair biuret, slight tyrosin, no tryptophane.
- With phenol-phthalëin = 6.8 c.c.  $\frac{N}{10}$  NaOH.
- With phenol-phthalëin = 0.1 c.c.  $\frac{N}{10}$  NaOH.
- Fibrin gone; very strong biuret, medium tyrosin, no tryptophane.
- 0.5 g fibrin; fair biuret, very slight tyrosin, no tryptophane.
- 0.37 g fibrin; fair biuret, very slight tyrosin, no tryptophane.

Starch and cane sugar were not tried in this experiment because of presence of hydrocyanic acid. No. 7 smelt strongly of acetic acid, showing that the titration figure was due to splitting of the methyl acetate. The fact that no tryptophane was found in No. 9 whilst markedly present in No. 1 might be due to there being two ferments, one splitting fibrin to the peptone stage and the other splitting peptones to amido-acids.

#### CHEMICAL EXAMINATION OF THE GLAND

A chemical examination of the gland was made in *Cancer pagurus*, *Patella*, and *Fusus*. The method employed was to mince the gland and extract with alcohol. After several extractions, the residue was next extracted several times with ether, and the ethereal extracts added to the alcoholic extracts. The alcohol and ether were evaporated off and the solid residue taken up in fresh dry ether. This was called the ethereal extract. The residue left after the removal of the ether soluble portion was treated with absolute alcohol and the solution called the alcoholic extract.

The portion left after treatment with alcohol consisted of substances soluble in water but which had dissolved in the first instance because the alcohol was diluted by the water contained in the tissues ; this was taken up in distilled water and called the watery extract.

*Cancer pagurus* : Ethereal extract = 8.53% of the fresh gland, golden-yellow colour, due to lipochrome.<sup>1</sup> This was redissolved in ether and precipitated by excess of acetone to find if any lecithin were present. The acetone precipitate and the fraction not precipitated by acetone were weighed, after drying, and saponified by alcoholic potash. The portion not precipitated by acetone consisted entirely of neutral fat, whilst the acetone precipitate gave a higher saponification value that would correspond to the weight of the material if it were considered to consist of neutral fat. After incineration the acetone precipitate showed the presence of phosphates.

1. Miss Newbiggin, *Journ. Physiol.*, Vol. XXI, p. 237, 1897.

Neutral fat = 7.77% of fresh gland, whilst acetone precipitate = 0.77% of the same.

Alcoholic extract = 0.32% of fresh gland.

Watery extract gave following reactions : pink biuret, tyrosin, faint tryptophane, no sugar, creatinin. No bile pigments by Gmelin's test, no bile acids by either Pettenkoffer's or Oliver's tests.

*Patella vulgata* : Ethereal extract = 7.49% of the fresh gland, dark greenish brown mass which gives when dissolved in alcohol the absorption bands of enterochlorophyll.<sup>1</sup>

Alcoholic extract = 0.81% of the fresh gland.

Watery extract shows biuret, tryptophane, very faint tyrosin and creatinin, some reduction of Fehling's solution. Gmelin and Pettenkoffer tests negative, but slight cloudiness with Oliver's reagent for bile acids.

*Fusus antiquus* : Ethereal extract golden brown (probably due to a lipochrome), with no absorption bands. Gives a precipitate with acetone which yields tests for phosphates after incineration.

Watery extract : Creatinin and tyrosin present, no biuret tryptophane or reduction of Fehling. Gmelin's test negative. Faint cloudiness with Oliver's reagent.

The glands show evidence of the products of digestion (albumoses, tyrosine and tryptophane). A large amount of fat and some lecithin. No bile pigments or acids but the faint cloudiness with Oliver's reagent suggests the presence of some substance in the mollusca which, like bile acids, is capable of precipitating albumoses. The presence of 'enterochlorophyll' in *Patella* (herbivorous) whilst it is absent in *Fusus* (carnivorous), supports MacMunn's view that enterochlorophyll is an indirect derivative of the chlorophyll contained in the animal's food.

Finally, I wish to express my thanks to Professor Herdman for his assistance and advice during the course of this research, and also to Professor Vines, of Oxford, for advice in connection with the digestion experiments.

1. Miss Newbiggin, *Q. J. M. S.*, Vol. XLI, p. 391, 1898, and MacMunn, *loc. cit.*

VARIATIONS IN THE HYDROCHLORIC ACID OF THE  
GASTRIC CONTENTS IN CASES OF CARCINOMA IN  
MAN

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(Received July 18th, 1906)

In the Proceedings of the Royal Society (Series B., Vol. 76, p. 138) Professor Moore published a series of observations which he, together with Messrs. Alexander, Kelly and Roaf, had made on the gastric contents of patients who were suffering from carcinoma situated in various parts of the body. As the result of these observations they came to the conclusion that the well known absence of free HCl in Carcinoma Ventriculi was due to the presence of Carcinoma in the body rather than to its presence in the stomach itself, for they found that the free HCl was very greatly diminished wherever the carcinoma might be situated in the body.

Working on the same lines and using the same methods,<sup>2</sup> except in a few small details, I have examined fourteen malignant cases and seven non-malignant cases ; my results agree very closely with those obtained by Moore and his colleagues and are, I think, worthy of publication as further evidence in support of their conclusions.<sup>3</sup> It will be seen in my deductions that the examination of non-malignant cases shows that the effect of diet and residence in a surgical ward considerably influences the gastric secretion and may be largely responsible for the diminution of free HCl in malignant cases.

1. The expenses of these experiments were defrayed by Dr. Pembrey from a sum granted by the Royal Society.

2. Further details and a review of the literature of the subject will be found in a paper to be published in the next volume of the Guy's Hospital Reports, Vol. LX.

3. In a paper published in *The Bio-Chemical Journal* for June, 1906, Moore, Alexander, Kelly, and Roaf incorporate the cases discussed in their previous paper of May, 1905, with a further series of cases. They also point out the influence of diet, etc., on the resulting estimations. My thesis for the M.D. degree, of which this paper is an abstract, was submitted on May 24th, 1906, and accepted on June 14th.

I have also endeavoured to discuss this question from its clinical aspect and have given details of my cases to enable some idea to be formed of the relation of the acidity to the condition of the malignant disease.

#### ROUTINE OF EXAMINATION

Before the patient was given his test meal he fasted for 14 hours, as by this means it was assured that the stomach was empty; unless, indeed, its mobility were very much impaired, an unlikely event as the patient examined complained of no gastric symptoms. A light supper consisting of bread and butter and milk or soup was given at 6 p.m., and nothing more was allowed till 8 a.m. the following morning, when the patient was given a slice of buttered toast and a pint of weak tea with a few drachms of milk and a little sugar. This differs from the ordinary test meal given by Ewald in containing more proteid, as he gave no sugar, milk or butter. As my purpose was to estimate the active HCl in the gastric juice and, as Pawlow has demonstrated that plain bread produces a much smaller flow of gastric juice than does proteid, in order to conduct the experiment under more advantageous conditions, I considered it advisable to introduce some proteid into the meal. I avoided, however, the heavy proteid meals advised by some observers, as the organic bases so introduced into the stomach combine readily with the HCl present.

Another of Pawlow's discoveries also influenced me in the choice of a test meal. He proved that gastric secretion varies directly with appetite, as was demonstrated by his well known experiment of feeding a dog by suggestion. Now a more unappetising diet than dry bread and milkless tea cannot possibly be imagined, but the addition of butter and milk increased the patients' appetites and so, therefore, their gastric secretion.

After an interval of an hour and a quarter to an hour and a half the stomach tube was passed, and some of the gastric contents siphoned off. In the majority of cases the mere act of passing the tube was sufficient to cause a gastric contraction which set up a siphon action which, once started, continued for about a minute. It is quite

unnecessary to remove the products of digestion by any aspiration or 'expression,' but occasionally it is difficult to start the syphon action and then a Higginson syringe, with a glass tube fitted into the receiver, can be applied for a few seconds and gently worked until the gastric contents begin to flow in the tube, when the syringe should be at once detached.

The fluid so obtained generally contained but little mucus, and the solid contents were usually finely divided. They were filtered through ash free filter paper—the ash of ordinary filter paper being capable of taking up some of the free HCl. For example, 20 c.c. of a solution of HCl were passed through an ash free paper, when it was found that 5 c.c. required  $3\cdot59 \frac{N}{100}$  NaOH to neutralise; on the experiment being repeated with an ordinary filter paper, 5 c.c. required only  $3\cdot41 \frac{N}{100}$  NaOH; thus, for every 5 c.c. passed through it, the ordinary filter paper absorbed  $\cdot18$  c.c.  $\frac{N}{100}$  more than the ash free paper.

The effective acidity of this fluid was then estimated by measuring the concentration of hydrogen ions present in the solution. This was done by means of the methyl acetate method which estimates the amount of acetic acid produced from the methyl acetate in a given time when it is in the presence of the gastric contents. This method was used by Moore and his colleagues in their investigations, and I have closely followed the technique described by them in their paper in the Proceedings of the Royal Society.

It is not necessary to further describe this technique but I shall point out a few differences which I introduced in the course of my investigation. Moore used a temperature of  $45^{\circ}$  for the thermostat in which he kept his solutions, and Hoffmann, in his original paper, used higher temperatures still; but the higher the temperature the greater the evaporation, and so I used a temperature of  $37^{\circ}$  C., at which the evaporation is almost nil, and thus avoided the necessity for making up the solution to its original volume before titration. Moreover, it is probable that at higher temperatures some of the HCl in combination with proteid is dissociated therefrom, and goes to swell the total free HCl.

As the fluid was yellow or brownish in colour some difficulty was

experienced in judging the exact point of neutralisation, so control solutions were used for comparison in each titration, and as sufficient fluid was not always obtained to provide these controls, the fluid had occasionally to be diluted exactly before estimation, and the results multiplied accordingly. In the table the figures given for titration in cases V, VI, VIII, XV are those actually obtained, and the values of C in the next column, the values for the original solutions, are obtained by multiplying those figures accordingly.

Sex	Age	Disease	Period of Digestion in Hours	Titration with Decinormal Soda and Phenol-phthalein			Period of Incuba- tion = t	Constant of Velocity of Reaction $10^6 C$	Effective Acidity in HCl percentage
				Initial	Final	Difference = x			
I	M.	56	Epithelioma Linguae	1 $\frac{1}{2}$	3.3	9.54	6.24	5 $\frac{1}{2}$	39.820
II	M.	56	Epithelioma Linguae	1 $\frac{1}{2}$	53	653	123	5	.7469
III	F.	46	Carcinoma of Colon	1 $\frac{1}{2}$	2.31	3.237	.927	5 $\frac{1}{2}$	5.2061
IV	M.	61	Carcinoma Recti	1 $\frac{1}{2}$	2.188	2.397	.209	6	1.0596
V	F.	43	Carcinoma Mamiae	1 $\frac{1}{2}$	85	90	.05	4 $\frac{1}{2}$	.7441
VI	M.	66	Epithelioma of Lip	1 $\frac{1}{2}$	053	124	.071	5	2.1535 <sup>a</sup>
VII	F.	62	Carcinoma Mamiae	1 $\frac{1}{2}$	941	1.038	.097	5 $\frac{1}{2}$	.5195
VIII	M.	48	Epith. Floor of Mouth	1 $\frac{1}{2}$	57	1.507	.937	5	28.9490 <sup>a</sup>
IX	M.	64	Carc. of Oesoph.	1	44	44	.00	5	.0000
X	M.	62	Epithelioma Penis	1 $\frac{1}{2}$	194	33	.136	5	.82613
XI	M.	63	Epith. Floor of Mouth	1 $\frac{1}{2}$	2.012	2.026	.014	5	.0848
XII	M.	57	Epithelioma of Lip	1 $\frac{1}{2}$	1.68	1.77	.09	5	.5462
XIII	M.	59	Epithelioma Penis	1 $\frac{1}{2}$	1.643	1.694	.051	6	.25769
XIV	M.	57	Melanotic Sarcoma	1 $\frac{1}{2}$	2.702	6.118	3.416	6	18.6091
XV	M.	33	Pyo Nephrosis	1 $\frac{1}{2}$	254	.288	.034	4 $\frac{1}{2}$	1.0847 <sup>a</sup>
XVI	M.	44	Simple Stricture Recti	1	91	3.29	2.38	5 $\frac{1}{2}$	13.8050
XVII	F.	29	Chronic Mastitis	1 $\frac{1}{2}$	60	.66	.06	5	.3639
XVIII	M.	48	Conjunctivitis and Keratitis	1 $\frac{1}{2}$	3.17	4.8	1.63	6	8.5216
XIX	M.	29	F.W.M.P.	1	2.19	7.39	5.2	5 $\frac{1}{2}$	32.2898
XX	M.	28	A.S.M.P.	1	2.16	7.09	4.93	5 $\frac{1}{2}$	30.40321
XXI	M.	28	T.W.	1 $\frac{1}{2}$	2.39	6.69	4.3	6	23.9283
								Average	.0887087
			$\frac{N}{10}$ HCl	..	..	..	..	..	119.2543
									.365

1. The value of  $10^6 C$  here given is twice that obtained from preceding columns as contents were diluted twice.  
2. " " " " " five times " " " " " " " five times.

In obtaining the various values of the constant velocity of reaction I have used common logarithms instead of the Napierian logarithms used by Moore, so that all the figures in this column are lower than the corresponding figures obtained by him. The figures in the last column are, however, obtained in each case from the *ratio* of the figures in the preceding column and so correspond exactly to the figures obtained by Moore.

The actual titrations were performed with centi-normal soda, and the results expressed in terms of deci-normal.

#### DISCUSSION OF THE RESULTS

1. Of the thirteen cases of non-gastric carcinoma which were examined only two showed effective gastric acidity to a normal degree. They were a case of epithelioma linguae, in which it was above normal, and a case of epithelioma of the floor of the mouth, in which it was almost exactly normal—where normal is taken as equivalent to the mean of observations taken on three healthy young men.

2. Of the remaining eleven cases, one showed one-sixth the normal effective acidity ; while the rest were all below one-fourteenth, most were below one-fortieth, several were below one-hundredth of normal. Thus, in the majority of the cases examined, the effective acidity was very greatly reduced, almost as much as in cases of carcinoma ventriculi. I suspect, indeed, that if a similar series of cases of carcinoma ventriculi were examined by the same method the results would be almost identical.

This would point to the conclusion that the diminution of HCl in cancer of the stomach is due to the presence of carcinoma in the body, and not to the position of that carcinoma in the stomach itself. And so far as my results go they uphold the views of Moore and his colleagues, although exceptional cases occur. It is interesting to notice that in the first series of ten cases which they investigated by this method a case of cancer of the tongue gave the highest percentage, '01239, though some of our lowest results also came from cases of tongue and floor of mouth.

3. It is well known that psychical influences play a great part in gastric secretion. Appetite, in the case of Pawlow's dog, increased the secretion of HCl, and a worried man is so often a dyspeptic, that I thought for some time that in this condition lay the solution of the low results which Moore and I obtained. I thought that the dread of approaching operation, or the horror of cancer always present in the lay mind, might cause the diminution. But in case VIII, although the man was extremely worried and harassed, his free HCl was normal, while in case X, the free HCl was one-fortieth of normal, and yet never was there a happier patient or one more free from dread. Psychical influences alone do not seem to be the cause of the diminished secretion.

4. It is interesting to discuss the stage at which carcinoma affects the gastric secretion, and the evidence of my results shows that this change is not necessarily delayed till the later stages of the disease. Only four of my cases could be described as inoperable—I, III, IV, XI, and yet the free HCl in case I was more than normal, and in case III, though much less than normal, yet it was the third highest value obtained. On the other hand, case V, which gave one of the smallest estimations, exhibited a very small growth.

5. In only one case could cachexia be said to exist, and that was a case of oesophageal stricture, the gastric secretion from which contained no free HCl. The majority of patients were healthy so far as one could judge from external appearances, so that cachexia cannot be the direct cause of the diminution.

6. The only case of sarcoma which I examined was one of melanotic sarcoma, probably the most malignant form of sarcoma; and yet it showed nearly normal free HCl, or very much more than eleven out of the thirteen cases of carcinoma.

7. I also examined four patients who were suffering from various non-malignant surgical affections and in two of them obtained very low readings, as low as in most of the malignant cases, while the other two were also less than normal. These cases are not sufficient in number to justify definite statements, but they certainly impair the value of the investigation of stomach contents as an aid to diagnosis,

and suggest that the diet and life in a surgical ward lead to some diminution in the gastric secretion.

8. The almost complete absence of any symptoms of dyspepsia in all my cases was very striking, a phenomenon which Ewald explains by supposing a substitution of intestinal for gastric digestion.

#### SHORT DESCRIPTION OF CASES

- I Thomas H., 56. A large inoperable epithelioma involving both sides of the tongue. Irritation from a tooth had been noticed for 6 months, but the tumour only 2 months. Much glandular enlargement, but no cachexia or wasting, he looked healthy and had no dyspepsia.
- II William M., 56. A small epithelioma on anterior part of tongue which was easily removed. Six weeks' history—some glandular enlargement. No dyspepsia, does not think he has lost weight.
- III Hannah C., 46. Carcinoma of Hepatic Flexure of Colon involving Peritoneum, which it had been found impossible to remove on laparotomy a few weeks before her stomach contents were examined. She said she had wasted and become paler—slight dyspepsia.
- IV William H., 61. Large definite carcinoma of rectum for which a colotomy was subsequently performed. History of only 3 months' symptoms, and yet radical treatment was impossible.
- V Leah L., 43. A small carcinoma of breast without much glandular enlargement. Only 5 months' history. She looked very healthy and was not at all wasted. Slight dyspepsia.
- VI Thomas G., 66. A small epithelioma of lower lip, which had started as a pimple 7 months before. There were some glands which were found to be inflammatory, and the man was healthy and well nourished.
- VII Hester N., 62. A small carcinoma of the left breast giving a few small glands. 12 months' history. Not wasted.
- VIII Alfred E., 48. Extensive epithelioma involving floor of mouth, lower jaw and submaxillary glands. He was wasted, though there was only a four months' history, and very worried and nervous about his operation which was going to take place on the morrow.
- IX Charles B., 64. An oesophageal stricture which was considered to be malignant—the bougie passing about 12 inches. Swallowing had been painful for about six weeks, and he was very wasted.
- X Henry M., 62. A very large growth at end of penis, which had been growing for 12 months. There was no loss of weight and no cachexia. This man was particularly cheerful—assured me that it was not 'cancer' and that no operation was necessary.

XI William W., 63. Large epithelioma which involved most of the right side of the floor of his mouth and caused much difficulty in swallowing. Only two months' history, and yet it was inoperable.

XII Job T., 57. Fairly large epithelioma of the tongue, with a history of two months. There was no wasting and he was a healthy looking man. Operation refused.

XIII Edwin B., 59. A very large foul epithelioma at end of penis which had been growing for 6 months. There was no cachexia, but he had lost half a stone in 6 months. His general condition was good in spite of the growth, and he denied all symptoms of dyspepsia.

XIV Charles B., 57. A large melanotic sarcoma which had started growing from a mole on abdominal wall near umbilicus 12 months before, and had already given secondary deposits in the axilla. The man was very fit, not at all wasted, and had no dyspepsia.

XV Walter S., 33. A large pyonephrosis on the right side, which had produced haematuria for 4 months and frequency of micturition for 2 months. The kidney was thought to be malignant, but operation proved it to be a simple pyonephrosis—probably tuberculous. The man was very healthy in appearance and general condition—appetite good, no dyspepsia, no wasting, and a good colour.

XVI Richard W., 44. A simple stricture of the rectum, which had given symptoms for 13 years. He was very slightly dyspeptic and had lost 23 lbs. in 6 months.

XVII Emily F., 29. A case of mastitis following prolonged lactation, the tumour had suppurated and produced several indolent sinuses. Patient was very well, fat, and denied all dyspepsia. After scraping, the breast healed up very well.

XVIII Richard G. S., 48. A case of conjunctivitis and keratitis with corneal ulcers.

XIX, XX, XXI. Healthy young men leading an active life.

In the above cases the diagnosis was usually made by the examination of microscopical sections of the growth, but in those cases where it was not possible to examine them histologically, the surgeon's diagnosis has been given. I am much indebted to the Surgeons of Guy's Hospital for their kind permission to utilise the clinical material in their wards.

THE STAINING ACT: AN INVESTIGATION INTO THE  
NATURE OF METHYLENBLUE-EOSIN STAINING

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The various theories of the nature of the act of staining of animal cells, tissues and products, which have been put forward by different writers fall into three groups: (1) that the process of staining is a chemical reaction between the dye on the one hand, and the cell or tissue elements on the other; (2) that in staining the dye is adsorbed by the tissue elements stained; and (3) that the dye is present in the form of a solid solution in the stained material. The problem remains, however, still unsettled, further data respecting individual stains being much to be desired.<sup>1</sup>

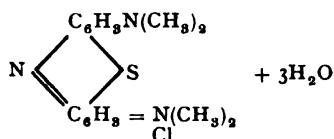
For studying the nature of an individual staining act methylenblue-eosin was selected as being a dye which is peculiarly suitable for the purpose. This substance is a salt which is formed by the union of the basic radical of methylenblue and the acid radical of eosin. When it is allowed to act upon cells or tissues some of the elements stain blue, others red. For example, with an alcoholic or aqueous solution of methylenblue-eosin a polynuclear leucocyte exhibits blue staining of the chromoplasm of the nucleus, while at the same time the oxyphile granules of the cytoplasm stain deep red, precisely similar blue staining being obtained with methylenblue and the red staining with eosin. In the case of methylenblue-eosin, provided that hydrolysis of the dye can be excluded, the act of staining can only be explained as due to a chemical reaction between dye and cell elements. If hydrolysis is not avoided then both adsorption and solid solution of the free acid and the free base may possibly occur and produce the staining effect.

1. Cf. W. M. Bayliss, 'On some aspects of adsorption phenomena,' *The Bio-Chemical Journ.*, 1906, Vol. I, p. 175.

As it seemed not improbable that an investigation of the colour and spectroscopic characters of methylenblue-eosin might throw light upon the presence or absence of hydrolysis of the dye, this was first undertaken. In this investigation the condition of methylenblue-eosin in solution in alcohol forms the primary object of study, but attention is also given to various points respecting alcoholic and watery solutions of methylenblue, eosin and allied substances, since these data have a direct or indirect bearing on the subject of enquiry. It will be convenient to commence with the latter two dyes.

#### COLOUR AND SPECTROSCOPIC CHARACTERS

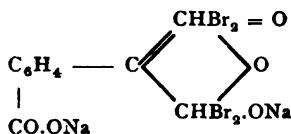
Methylenblue is tetramethyldiamidothiodiphenylamin chloride, having the formula



and the molecular weight 371, or after freeing from water at 100° C., 317.36. This salt crystallises in dark blue microscopic plates; from solution in water it is precipitated by sodium chloride and by zinc chloride. The form employed in the present work was the double salt with zinc chloride, having the formula  $2\text{C}_{16}\text{H}_{18}\text{N}_2\text{SCl} + \text{ZnCl}_2 + \text{H}_2\text{O}$ , and the molecular weight 787.96, or when anhydrous 770.08. The sample employed (rectif., for injection *intra vitam*, Grüber) contained 9.12% s. (theory requiring 8.17%). Methylenblue, if allowed to separate out slowly from alcoholic or aqueous solution crystallises in needles and prisms having a bronze lustre on the surface, but when rapidly evaporated it forms a film, which under the microscope does not exhibit crystals, but is amorphous in character. In alcoholic and in aqueous solution of 0.001 N to 0.0001 N concentration (0.0079% to 0.00079%) it forms a characteristic blue liquid, no difference of colour between alcoholic and aqueous solutions being recognisable to the naked eye, though on spectroscopic examination a marked difference is recognisable. Thus in alcoholic solution of  $2.80 \times 10^{-3}$  N

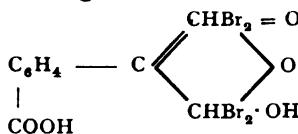
concentration, in a layer 1 cm. thick, a very dark band appears in the red, while between this band and the solar line D a lesser degree of absorption occurs (Fig. 1, A) ; in aqueous solution a narrower very dark band is seen and a second less dark band running up to D (Fig. 1, H).

Water soluble eosin, the sodium salt of tetrabromfluorescein,  $C_{20}H_6Br_4O_5Na_2 + 5H_2O$ , to which the constitutional formula



has been assigned,<sup>1</sup> has a molecular weight of 776.2 or, without water of crystallisation 686.2. The sample employed contained after drying 41.7% Br, theory requiring 47.2% Br. From watery solution it crystallises out in triclinic red crystals, exhibiting a greenish surface lustre. Its colour in 0.01 N concentration (0.078%) in alcohol is yellowish red with a strong light-yellowish green fluorescence ; in water it is of a darker brownish red colour and shows a slighter olive green fluorescence. In 0.0001 N concentration the same difference in respect of fluorescence is seen, but the colour in alcohol is a vivid pink, in water a slightly brownish pink.<sup>2</sup> The spectrum in alcohol in  $2.45 \times 10^{-5}$  N concentration, in a layer 1 cm. thick shows a very dark band in the green about the solar line E, together with a second lighter band towards F, as shown in Fig. 1, B ; in aqueous solution of the same concentration a broader very dark band nearer the blue end of the spectrum is seen, but no second absorption band is observed (Fig. 1, F).

Alcohol soluble eosin ( $C_{20}H_6Br_4O_5$ ), the free acid corresponding to the foregoing salt, receiving the formula



1. The exact constitution of the eosin molecule is not yet established. In accordance with current views of the nature of the chromophore group, the quinone ring is exhibited in the formulae given.

2. The above statements refer to the colour of solutions in test-tubes. A layer of eosin solution of 0.01 N concentration, 5 mm. thick, has the same colour as a layer of eosin solution of 0.0002 N, 25 mm. thick.

has a molecular weight of 643.0. The sample used contained after drying 47.6% Br, theory requiring 48.3% Br. From alcoholic solution it crystallises on slow evaporation in yellowish red crystals,

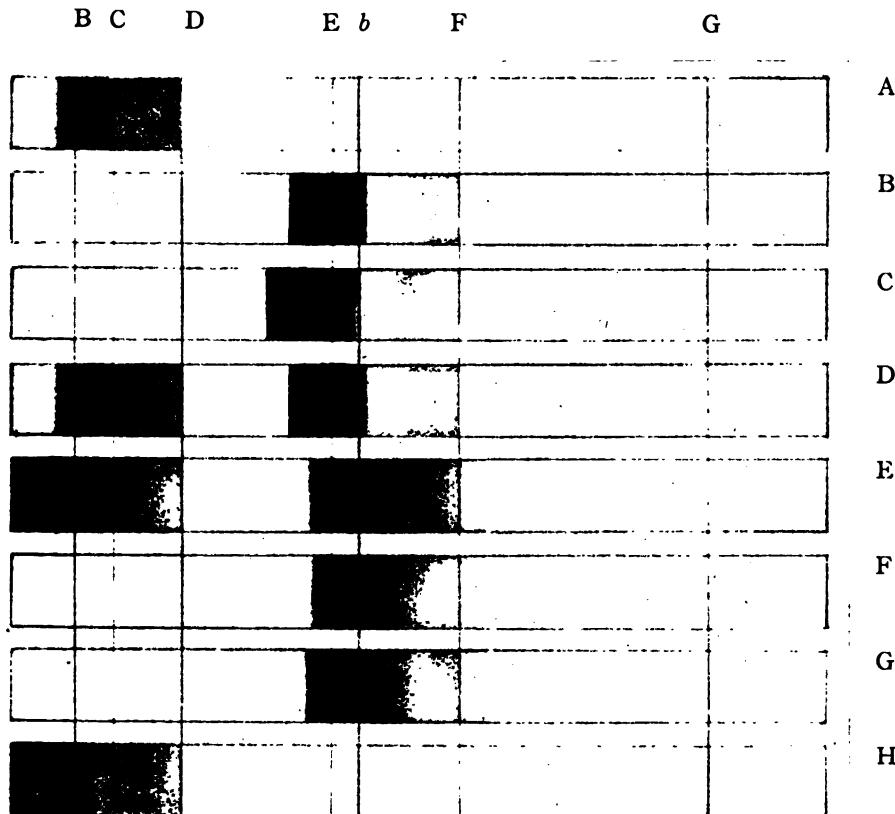


Fig. 1. ABSORPTION SPECTRA. In absolute alcohol : A, methylenblue, in  $2.78 \times 10^{-6}$  N concentration ; B, water soluble eosin, in  $2.45 \times 10^{-6}$  N concentration ; C, alcohol soluble eosin, in  $2.50 \times 10^{-6}$  N concentration ; D, methylenblue-eosin, in  $2.80 \times 10^{-6}$  N concentration. In water : E, methylenblue-eosin, in  $2.80 \times 10^{-6}$  N concentration ; F, water soluble eosin, in  $2.45 \times 10^{-6}$  N concentration ; G, alcohol soluble eosin, in  $2.50 \times 10^{-6}$  N concentration ; H, methylenblue, in  $2.78 \times 10^{-6}$  N concentration. The liquids were viewed in a layer 1 cm. thick.

having the formula  $C_{20}H_8Br_4O_6C_2H_6O$ ; on rapid evaporation a film forms in which no crystals are recognisable; from alcoholic solution to which hydrochloric acid has been added, in red alcohol-free

erystals. The colour of a '001 N solution in alcohol is a deeper pink than that of the sodium salt in the same solvent, and is accompanied by a slight yellowish green fluorescence; in '0003 N concentration in water its colour is a deeper brownish pink with a slight olive-green fluorescence. In alcohol in '00001 N concentration it shows a brilliant slightly violet pink colour, and in water in the same concentration a slight brownish tint is added, the same difference as before in respect of fluorescence being seen. The spectrum of alcohol soluble eosin of  $2.50 \times 10^{-5}$  N concentration in a layer 1 cm. thick in alcohol (Fig. 1, C) shows a very dark broad band in the green and to the side of this, nearest the blue end of the spectrum, a second lighter narrower band, both bands being slightly nearer the red end of the spectrum than the corresponding bands of water soluble eosin, whose dark band is not so wide. In aqueous solution in the same concentration (Fig. 1, C), the absorption spectrum differs from that of the sodium salt in water in the same concentration (Fig. 1, F), in the diminished width of the dark band which is displaced towards the red, an exceedingly faint second absorption band being seen towards F.

Methylenblue-eosin was first prepared by Romanowsky<sup>1</sup> (1891), who observed that when concentrated solutions of methylenblue and eosin were mixed a precipitate formed, which, when dissolved, furnished a selective stain for the nuclear chromoplasm of the Plasmodium malariae. Ziemann<sup>2</sup> (1898), who made use of the same stain, observed that the precipitate was soluble in excess of the methylenblue, as also of the eosin solution. Rosin<sup>3</sup> (1899), found that similar precipitates were obtainable from mixtures of other so-called basic and acid dyes. None of these observers, however, investigated the chemical composition of Methylenblue-eosin. Jenner<sup>4</sup> (1899), who made use of this dye for staining blood-films, found that it dissolved to the extent of about 5% in the methyl alcohol, was less soluble in ethyl alcohol, and

1. *Zur Frage der Parasitologie und der Therapie der Malaria*, 1891.

2. 'Über Malaria und andere Blutparasiten,' etc., Jena, 1898, Fischer. Also *Centralbl. f. Bakteriol.*, 1898, No. 25.

3. 'Über eine Gruppe von Anilinfarbstoffen, ihre Bedeutung für die Biochemie der Zelle und ihre Verwendbarkeit für die Gewebsfärbung,' *Berl. kl. Wochenschr.*, 1899, 36 Jahrg., p. 251.

4. 'A new method for rapidly fixing and staining blood,' *Lancet*, 1899, Vol. I, p. 370.

much less soluble in water<sup>1</sup>. He regarded methylenblue-eosin as a salt, since he observed that, no matter what the proportions in which methylenblue and eosin were mixed might be, the precipitate formed always possessed the same melting point, namely, 227° C. The precipitate, he further observed, consisted of brilliant grass-green crystals if alcoholic solutions were used (with rapid evaporation a film forms in which no crystals are recognisable), but was amorphous and showed a metallic green colour if aqueous solutions were employed. Jenner did not determine the composition of methylenblue-eosin, and though he mentions that the most abundant precipitate is formed when methylenblue and eosin are mixed in the proportion of 8·0 to 8·3 parts of the former and 10 parts of the latter, yet this statement, in the absence of further details as to the composition and purity of the methylenblue and eosin employed, does not enable a conclusion to be reached as to the composition of the precipitate. The absence of a sharp indication of accurate admixture of methylenblue and eosin renders this method of attempting to determine the relative proportions required unsatisfactory. It may be pointed out that if the proportion of two molecules of methylenblue ( $2C_{18}H_{18}N_3SCl + ZnCl_2 + H_2O$ , mol. wt. 788) and one of eosin ( $C_{20}H_6Na_2Br_4O_5 + 5H_2O$ , mol. wt. 776) are employed, the ratio of the former to the latter, by weight, would be 10·2 : 10; if the proportion is one and a half to one, the ratio becomes 7·55 : 10.

The composition of methylenblue-eosin<sup>2</sup> was therefore further investigated, two methods being employed. In the first place by means of the colorimetric observations described below it was ascertained that the proportions in which methylenblue and eosin react to produce methylenblue-eosin were two molecules of the former ( $C_{18}H_{18}N_3SCl$ ) and one molecule of the latter ( $C_{20}H_6Na_2Br_4O_5$ ). In the second place, by ultimate analysis, the percentages of sulphur and bromine respectively in methylenblue-eosin dried to constant weight at 100° C. were found to be 5·12 and 23·50, a second analysis giving

1. It dissolves in water to the extent of about 0·02 per cent. at 16° C.

2. Methylenblue-eosin is quite free from chlorine and zinc on the one hand, and from sodium, potassium and ammonium on the other, so that the possibility of its being an admixture of methylenblue and eosin is altogether excluded.

5.35 and 23.84. The formula  $(C_{16}H_{18}N_3S)_2C_{20}H_6Br_4O_5$  (mol. wt. 1204.4), which requires 5.28% of sulphur and 26.34% of bromine, therefore represents the required relation.

The colour of methylenblue-eosin in absolute alcohol in 0.01 N concentration (12%) is dark purple with a strong dark yellowish green fluorescence; in 0.0001 N concentration, bluish purple with the same strong fluorescence. In aqueous solution in the latter concentration its colour is reddish purple with a very slight dark olive green fluorescence. In alcoholic solution of  $2.8 \times 10^{-5}$  N concentration in a layer 1 cm. thick, methylenblue-eosin exhibits a spectrum (Fig. 1, D) showing absorption bands both in the red and the green, the former being identical with that of methylenblue in corresponding concentration (Fig. 1, A), the latter with that of water soluble eosin in corresponding concentration. In aqueous solution in the above concentration and thickness, a different absorption spectrum is seen, in the red a very dark band touching Fraunhofer's line B being seen with a lighter absorption area on each side; while in the green a very dark band centred about b is seen, with a lesser degree of absorption towards F but without a second lighter band as in alcohol. Methylenblue-eosin, therefore, in water shows bands which are different from those exhibited by methylenblue and eosin respectively in aqueous solution.

If methylenblue-eosin is dissolved in a test-tube of alcohol in amount sufficiently small to produce a convenient depth of colour<sup>1</sup>, no difficulty is experienced in ascertaining the respective amounts of methylenblue and of water soluble eosin, which require to be added to a second tube of alcohol in order to produce the same quality and intensity of colour, which the first exhibits. If the eosin is employed in the alcohol soluble form, the colour of the first tube can be very nearly matched but complete equality of colour is not attainable. In water, also, a methylenblue-eosin solution may similarly be matched with methylenblue and water soluble eosin, but when alcohol soluble eosin is employed matching is attended with difficulty, for although

1. Only dilute solutions can be employed owing to the intense colour of the dyes. The concentrations given in Table II will be found suitable in most cases.

immediately after mixing the latter two dyes in suitable proportions a fair equality of colour is obtainable yet the colour rapidly changes, the mixed liquid becoming bluer until at the end of twenty-four hours it has assumed the appearance of a methylenblue solution, the eosin having separated out in the form of a red precipitate, which has settled at the bottom of the test-tube. It appears, therefore, that in this case methylenblue-eosin is not formed, a simple mixture of the two dyes resulting and the alcohol soluble eosin being after a time 'salted out' by the methylenblue. In two cases, however, namely when methylenblue and water soluble eosin are employed in alcohol or in water, equality of colour is attainable both as regards tint and depth. The smallest variation of colour which, under the most favourable conditions in respect of light, of background and of freedom of the eyes from fatigue, is recognisable with certainty is represented by a variation of about  $2\frac{1}{2}\%$  in the amount of the solutions of methylenblue or water soluble eosin required to produce equality. When equality of colour has been attained the spectra are found to be identical also. The concentration of methylenblue and of water soluble eosin, which was found after repeated trials to give equality, is shown in Table 2, Exps. 1 and 2. The composition of the methylenblue-eosin employed has been already referred to (p. 411); the sample of methylenblue,  $2(C_{16}H_{14}N_3SCl) \cdot ZnCl_2$ , used was found on analysis (p. 407) to contain 9.12% of sulphur (theoretically 8.17% is required), and the eosin salt,  $C_{20}H_6Br_4O_5Na_2$ , contained (p. 408) 41.7% of bromine (theoretically 47.20%). It is clear, from the results in Table 2, that in the formation of methylenblue-eosin two molecules of zinc-free methylenblue,  $C_{16}H_{14}N_3SCl$ , and one molecule of eosin take part. It is to be noted that the slight defect of the eosin radical already noted in the methylenblue-eosin employed has its counterpart in the concentrations required to match ( $48 \times 10^{-5}$  N instead of  $54 \times 10^{-5}$  N, Table 2, Exps. 1, 3, 5, 7). The cause of these differences of concentration obtaining when equality of colour was reached is presumably to be attributed to the formation of small amounts of leuco or other derivatives of feeble colour intensity; no indication of the presence of such compounds is, however, afforded by the analyses made (pp. 407-

411). It would appear that methylenblue and methylenblue-eosin are more liable to deteriorate on keeping than is eosin.

If, instead of adding methylenblue and eosin to the same liquid these dyes are dissolved separately in the concentrations given above and are placed in glass cells 3 cm. thick, one in front of the other, the transmitted light can then be compared with that of a solution of methylenblue-eosin of similar concentration contained in a third cell of the same dimensions as the former. Examined in this way in alcoholic solution the colour of methylenblue-eosin is found to be accurately matched, when the same concentrations are employed as in the test-tube experiment, if eosin in the form of the sodium salt is employed ; if, however, eosin in the form of the free acid is used, the colour of the apposed solutions, though approximating very nearly to that of the solution of methylenblue-eosin is not quite identical with it (Table 2, Exps. 2 and 4). When watery solutions are employed, the colour of the apposed liquids is too blue compared with that of the single liquid, whichever form of eosin is used, the same concentrations being employed as for the test-tube experiments above described ; if, however, the concentration of the eosin is sufficiently increased (Table 2, Exps. 7 and 8), then although a very slight increase or diminution of the concentration of the eosin causes the colour of the apposed cells to become too red or too blue, nevertheless an absolute identity of colour is not attainable. In this connection reference should be made to Table 1, in which an attempt is made to exhibit the relative colour of the dyes employed in a form suitable for ready comparison.

If the spectra of the apposed liquids are studied (Fig. 1), it is found that in all cases the spectra are the sum of those of the individual liquids, as is to be expected, when it is borne in mind that eosin has no absorptive effect whatever at the red end of the solar spectrum, nor does methylenblue absorb any light in the central part of the solar spectrum. It will now be readily understood that identity of colour is obtainable only when methylenblue-eosin is matched with methylenblue and water soluble eosin, alcohol being the solvent employed. The spectroscope thus affords a valuable means of checking and con-

firmering the conclusions arrived at by the eye. The approximation to equality attained by increasing the strength of apposed watery solutions of eosin (Table 2, Exps. 7 and 8; cp. Fig. 1, D to F) is reached by increasing the absorption of light towards the solar line F, but no true equality of colour is possible.

Before proceeding to consider the bearing of the above data upon the nature of the staining act of methylenblue-eosin, it will be of advantage to make a brief reference to the hydrolysis of organic salts. Water, which possesses a very high dielectric constant and at the same time exhibits an extremely low degree of ionisation, has the property of hydrolysing salts formed by the combination of (1) a weak base with a strong acid, or (2) a weak acid with a strong base, or (3) a weak base with a weak acid. Thus anilin hydrochloride in  $0.3$  N concentration is hydrolysed to the extent of  $2.6\%$ , while urea hydrochloride in the same concentration is hydrolysed to as much as  $95\%$ <sup>1</sup>; potassium cyanide in  $0.2$  N concentration is hydrolysed to the extent of  $2.3\%$ <sup>2</sup>; anilin acetate in  $0.1$  N concentration is hydrolysed to the extent of  $55\%$ .<sup>3</sup> From these illustrations which can be multiplied it is obvious that, before attempting to explain the nature of methylenblue-eosin staining it is essential to ascertain how much this dye is hydrolysed by the solvent employed.

In solution in alcohol the spectroscopic characters of methylenblue-eosin show that this dye is not hydrolysed into methylenblue (free base) and eosin (free acid) to any recognisable extent, for the absorption spectrum of methylenblue-eosin in the green coincides with that of water soluble eosin, and no modification due to the presence of alcohol soluble eosin can be detected (Fig. 1, A to D). The question now arises, what is the smallest amount of alcohol soluble eosin, the addition of which (in an apposed cell) can be recognised spectroscopically. This addition may be put at about  $25\%$  of the concentration of the methylenblue-eosin examined ( $2.8 \times 10^{-5}$  N), when the spectroscopic appearance is observed, but when the colour

1. Shields (1893), *Zeitschr. f. physik. Chem.*, Bd. XII, s. 167; Walker (1899), *ibid.*, Bd. XXXII, s. 137.

2. Walker (1889), *Zeitschr. f. physik. Chem.*, Bd. IV, s. 319.

3. Arrhenius and Walker (1890), *Zeitschr. f. physik. Chem.*, Bd. V, s. 18.

is judged with the naked eye an addition of 10%, or under extremely favourable circumstances of 2½%, can be recognised. If methylenblue (with or without the addition of an equivalent amount of potassium hydroxide), or alcohol soluble eosin is employed in alcoholic solution in one-tenth of the concentration in which methylenblue-eosin is ordinarily used (*i.e.* '005 N or '6%)<sup>1</sup> for staining purposes in alcohol, the respective blue and red differential staining cannot be obtained or is exceedingly faint ; to obtain staining equal to that of methylenblue-eosin in the same period of time, the concentration must equal or, better still, exceed that of the methylenblue-eosin stain. Since, therefore the latter dye in alcoholic solution is not hydrolysed to an extent sufficient to furnish a possible explanation of its staining power, the conclusion follows that its staining act is a chemical reaction in which the dye molecules are broken up, and not a purely physical process. It is interesting to observe that no recognisable hydrolysis of water soluble eosin in  $2.5 \times 10^{-5}$  N concentration in alcohol is obtainable, for if sodium hydrate dissolved in absolute alcohol is added no change of colour or spectrum can be detected. Owing to the relatively low value of the dielectric constant of alcohol (26 for ethyl alcohol) compared with that of water (80) ionisation of the dyes need not be taken into account.

In water, methylenblue-eosin presents an absorption spectrum in the green (Fig. 1, H) differing slightly but distinctly from that of water soluble eosin (Fig. 1, G), while its absorption spectrum in the red differs from that of methylenblue in the form of chloride (Fig. 1, E) or of free base (Fig. 2, x). Since the absorption spectrum of methylenblue-eosin is not compounded of that of methylenblue and water soluble eosin, colour matching by apposition is not possible as in the case of alcoholic solutions, and the only means of judging of the existence of hydrolysis is by means of spectroscopic examination. Unfortunately this, while failing to afford evidence of eosin in the form of free acid, is not sufficiently delicate (*cp. p. 413*) to afford proof that hydrolysis, if present, would be in so small a degree that the possibility

1. In methyl alcohol (Jenner's stain). In water the concentration is usually about one-fifteenth of this amount.

of the red and blue staining being a physical process would be altogether excluded. The colour and spectrum of methylenblue chloride in water, it may be observed, do not differ from those of the free base in water (Fig. 2, x). The spectrum of water soluble eosin in  $2.5 \times 10^{-5}$  N concentration in water affords no evidence of hydrolysis, for on adding sodium hydrate no change is produced; in the stronger solution used for staining (0.005 N) the degree of hydrolysis must be insignificant, since the degree of hydrolysis is inversely proportional to the square root of the concentration.<sup>1</sup> It does not, however, follow that the production of alcohol soluble eosin in watery solution of methylenblue-eosin would be relatively less in the 0.005 N concentration used for staining than in the concentration ( $2.8 \times 10^{-5}$  N) submitted to spectroscopic examination, for in the case of a salt formed, as is methylenblue-eosin by the combination of a weak base with a weak acid, the amount of hydrolysis occurring may be independent of the concentration.<sup>2</sup> In such a case also a marked degree of ionisation may be present. Thus anilin acetate in watery solution is not only hydrolysed to about 55% in all concentrations, but the undissociated fraction is nearly completely ionised in centinormal concentration. Ionisation might be imagined to account in part for the circumstance that the spectrum of methylenblue-eosin is not compounded of those of methylenblue and eosin (sodium salt). This may be so, but the data given in the next section indicate that such an interpretation is insufficient to afford a satisfactory explanation of the difference in question. A more probable explanation is that the chromophore radicals in methylenblue-eosin mutually modify each other, though it is to be noted that no such modification is to

1. According to the formula  $x = \sqrt{\frac{1}{c} \cdot \frac{k_w}{k_s}}$ , where  $x$  is the degree of hydrolysis,  $c$  is the concentration,  $k_w$  is the water constant ( $1.2 \times 10^{-14}$ ), and  $k_s$  the dissociation constant of the free acid of eosin. This applies only if  $k_s$  is much larger than  $k_w$ .  $K_s$  is unknown, but since water soluble eosin in aqueous solution is not precipitated by carbonic acid, it follows that  $k_s$  is not less than the dissociation constant of the latter ( $3 \times 10^{-7}$ ), so that the formula holds good for eosin.

2. The relation is  $\left(\frac{1-x}{x}\right)^2 = \frac{k_b k_s}{k_w}$ , where  $x$  is the degree of hydrolysis,  $k_b$  the dissociation constant of the free base of methylenblue,  $k_s$  that of the free acid of eosin, and  $k_w$  the water constant.

be observed in alcoholic solution. The presence or absence of ionisation is not of much importance in deciding the problem at issue, for the possibility of a physical as opposed to a chemical process occurring in methylenblue-eosin staining turns upon the occurrence of hydrolysis. It is interesting to observe that the addition of chlorine ions (*i.e.* of  $0 \cdot 13$  N NaCl) to an aqueous solution of methylenblue ( $6 \times 10^{-5}$  N) the effect of which would be to diminish ionisation of methylenblue, causes the second faint band in the red to become more distinct.<sup>1</sup> When the concentration of sodium ions is increased by adding sodium iodide to water soluble eosin in aqueous (or alcoholic) solution, the colour and spectrum undergo no immediate change (subsequently a slow change occurs requiring several weeks for its full development).

To sum up we may say that methylenblue-eosin in alcoholic solution is not perceptibly hydrolysed, and that its staining act is therefore a chemical, not a physical, process. The data at present available, however, do not enable a conclusion to be arrived at as to the nature of the staining act in aqueous solution.

Although direct proof of the nature of methylenblue-eosin staining in aqueous solution is not available, nevertheless the fact that the staining act in alcoholic solution is a chemical reaction implies, though it does not prove, that the same holds for aqueous solution. The circumstance that the blue staining of nuclear protoplasm is effected not only by methylenblue-eosin in both alcoholic and aqueous solution, no matter whether in the form of chloride or of free base, is not easy to explain as a physical process due to adsorption or selective solubility of the dye, because the physical conditions in the four cases are dissimilar; on the other hand on the theory of stoichiometric reaction no such difficulty is experienced, the process being in each case the same. The same may be said of the staining of the oxyphile granules of leucocytes by methylenblue-eosin in alcoholic and in watery

1. In alcoholic solution of methylenblue the addition of  $\text{CaCl}_2$  in  $2 \times 10^{-2}$  N concentration or of HCl in  $4 \times 10^{-2}$  N concentration does not alter the spectrum. In each of these cases advantage is taken of the relation  $\frac{\text{concentration of anion} \times \text{concentration of kation}}{\text{concentration of undissociated salt}} = \text{constant}$ . If the colourless ion is increased in amount the amount of coloured ion becomes reduced to insignificant dimensions and does not appreciably modify the colour of the undissociated dye.

solution, by alcohol soluble eosin in alcoholic solution, and by water soluble eosin in watery solution, all three dyes being in the same molecular concentration.

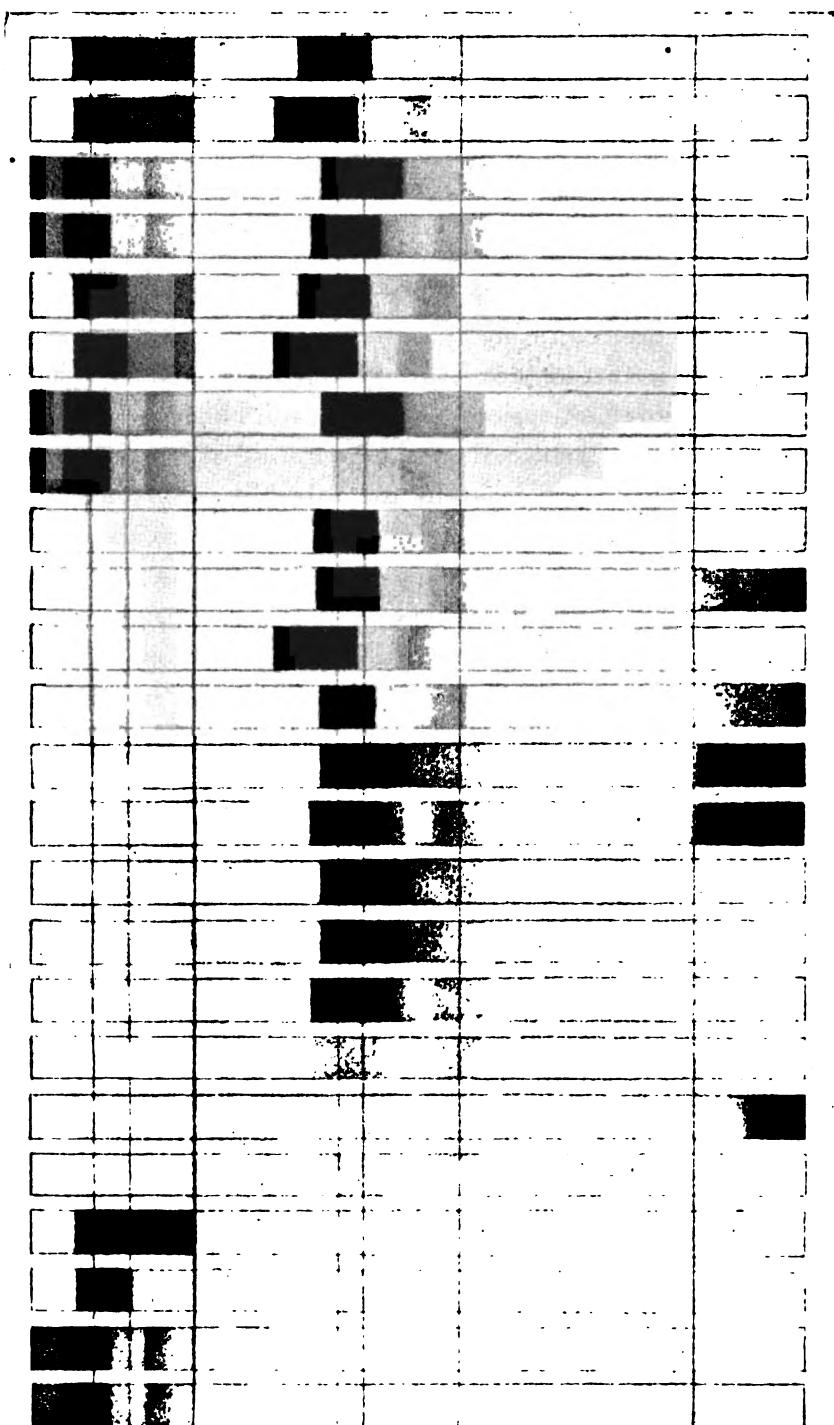
It is very desirable that the condition of the dyes employed should be further defined in respect of osmotic pressure and of ionisation. The latter cannot at present be determined with sufficient accuracy to be of much value owing to the impossibility of ascertaining with accuracy the fraction of the conductivity which is to be attributed to impurities in the form of electrolytes. Osmotic pressure will be dealt with in the next section, but before passing to this subject some further details respecting the colour and spectra of the dyes employed, in various conditions, will be given.

It has long been known that when an acid, mineral or organic, is added to an alcoholic solution of methylenblue-eosin the solution assumes the colour of methylenblue and on spectroscopic examination shows the spectrum of methylenblue (Fig. 1, A) unchanged while the eosin bands are more or less obliterated (as in Fig. 2, s, t), and when the mixed liquid is used for staining sections only the eosin colouration appears. If, instead of acid, an alkali is added, the solution turns reddish violet in colour and shows the eosin (Fig. 1, B) spectrum, while the methylenblue spectrum becomes modified (as in Fig. 2, v); on using the liquid for staining sections only blue staining is obtained. If this observation is performed in a test tube,  $2 \times 10^{-5}$  N will be found a convenient concentration for the dye, and the acid and alkali should be employed in  $1 \times 10^{-3}$  N concentration; if the latter are employed in  $5 \times 10^{-5}$  N concentration the acid changes the colour of the liquid, but no change is produced by the alkali. In aqueous solution, contrary to what would be expected, no change of colour occurs. Now the action of acid in alcoholic solution is obviously directed to the acid radical of methylenblue-eosin, for the addition of acid to methylenblue in solution does not alter the colour or spectrum of the solution. Similarly alkalies in alcoholic solution act upon the basic radical of methylenblue-eosin, for the addition of alkalies to the sodium salt of eosin produces no change of colour (only after prolonged boiling with concentrated alkali is a change of colour producible).

B C D

E b F

G



a  
b  
c  
d  
e  
f  
g  
h  
i  
j  
k  
l  
m  
n  
o  
p  
q  
r  
s  
t  
u  
v  
w  
x

Fig. 2. Spectra of various solutions viewed in a layer one centimetre thick, except when otherwise stated. The following spectra are each obtained from two liquids apposed in separate cells (Mb = methylenblue,  $\text{Na}_2\text{E}$  water soluble eosin,  $\text{H}_2\text{E}$  alcohol soluble eosin) :

- a. Mb  $2\cdot7 \times 10^{-5}$  N +  $\text{Na}_2\text{E} 2\cdot4 \times 10^{-5}$  N. Solvent—Absolute alcohol.
- b. „  $2\cdot6$  „ +  $\text{H}_2\text{E} 2\cdot5$  „ „ „
- c. „  $2\cdot7$  „ +  $\text{Na}_2\text{E} 2\cdot4$  „ „ „ Water.
- d. „  $2\cdot6$  „ +  $\text{H}_2\text{E} 2\cdot5$  „ „ „

The remaining spectra are each obtained from a single solution :

- e. Mb  $2\cdot7 \times 10^{-5}$  N +  $\text{Na}_2\text{E} 2\cdot4 \times 10^{-5}$  N. Solvent—Absolute alcohol.
- f. „  $2\cdot6$  „ +  $\text{H}_2\text{E} 2\cdot5$  „ „ „
- g. „  $2\cdot7$  „ +  $\text{Na}_2\text{E} 2\cdot4$  „ „ „ Water.
- h. „  $2\cdot6$  „ +  $\text{H}_2\text{E} 3\cdot0$  „ „ „
- i.  $\text{Na}_2\text{E} 2\cdot5$  „ +  $\text{Na I} 1\cdot7$  N. „ „ Absolute alcohol.
- j. „ „ „ +  $\text{Na I} 1\cdot7$  N. „ „ „
- k.  $\text{H}_2\text{E} 2\cdot5$  „ +  $\text{Na I} 1\cdot7$  N. „ „ „
- l. „ „ „ +  $\text{Na I} 1\cdot7$  N. „ „ „<sup>1</sup>
- m.  $\text{Na}_2\text{E} 2\cdot3$  „ +  $\text{Na I} 1\cdot1$  N. „ „ Water.<sup>1</sup>
- n.  $\text{H}_2\text{E}$  „ „ +  $\text{Na I} 1\cdot1$  N. „ „ „<sup>1</sup>
- o.  $\text{Na}_2\text{E}$  „ „ „ „ „ „
- p. „ „ „ +  $\text{NaCl} 1\cdot5$  N. „ „ „
- q.  $\text{H}_2\text{E}$  „ „ „ „ „ „
- r. „ „ „ +  $\text{NaCl} 1\cdot5$  N. „ „ „
- s. „ „ „ +  $\text{H}_2\text{SO}_4 1 \times 10^{-3}$  „ „ Absolute alcohol.<sup>2</sup>
- t. „ „ „ +  $\text{H}_2\text{SO}_4$  „ „ „ Water.<sup>2</sup>
- u. Mb  $2\cdot7$  „ „ „ „ „ Absolute alcohol.
- v. „ „ „ +  $\text{KOH} 1 \times 10^{-3}$  „ „ „
- w. „ „ „ „ „ „ Water.
- x. „ „ „ +  $\text{KOH} 1 \times 10^{-8}$  „ „ „

1. Spectrum observed after the liquid had been mixed for a week.

2. In a layer three centimetres thick.

If to an alcoholic solution of methylenblue (the most convenient concentration being that of  $1 \times 10^{-4}$  N to  $1 \times 10^{-5}$  N) sodium or potassium hydrate is added in the proportion of one molecule to one of methylene-blue, the liquid preserves its colour and shows no alteration of its spectrum ; if alkali is added in greater quantity, *e.g.* in the proportion of twenty molecules to one of methylenblue, the colour quickly changes to reddish-purple subsequently becoming purple-red, the methylenblue band in the spectrum showing less absorptive power between C and D while the dark band retains its position and intensity

undiminished, as in Fig. 2, *v*. In watery solution no change is produced either in colour or spectrum by the addition of alkali.

If to a dilute alcoholic or watery solution of eosin in the form of the sodium salt hydrochloric or sulphuric acid is added in equimolecular amount, the colour of the liquid changes and inclines to that of the acid eosin, the spectrum changing also to that of acid eosin, but the colours and spectrum are not quite pure for the further change next to be described is easily produced if too much acid is added.

Excess of acid (five to twenty molecules of acid to one molecule of eosin) almost completely decolourises eosin solutions of  $2.5 \times 10^{-5}$  N concentration, producing in alcohol a faint light brownish colour with an exceedingly faint fluorescence and the spectrum shown in Fig. 2, *s.*, while in water a light reddish-brown colour with a very slight olive-green fluorescence is seen, and the faint absorption spectrum, shown in Fig. 2, *t.*, is obtained. To observe these spectra a layer of fluid three or more centimetres in thickness is necessary.

If to a  $2.5 \times 10^{-5}$  N solution of water soluble eosin sodium iodide in  $1\text{ N}$  or greater concentration is added, the colour in alcoholic solution changes slowly to a slightly brownish pink and the spectral bands at E and F are slightly displaced towards the blue end, while a faint band appears beyond G; at the end of a week the solution has become of a darker yellowish brown colour and the latter band is deeper (Fig. 2, *i* and *j*). In water, the dye, after the addition of iodide of sodium retains its colour unchanged but loses its fluorescence, the bands at E and F being unaltered, but a dark band has appeared extending to the right of G.

If alcohol soluble eosin is employed, the addition of sodium iodide produces in alcoholic solution a slightly brighter and more violet-pink colouration, but no recognisable alteration of the spectrum is at first noted; at the end of a week a yellowish-brown colour is produced, and the spectrum is that shown in Fig. 2, *l*. In watery solution the colour does not change on adding sodium iodide, but fluorescence is abolished, the spectrum remaining unchanged; at the end of a week the liquid has become of a reddish-brown colour and exhibits the spectrum shown in Fig. 2, *n.*

If to an aqueous solution of water soluble eosin, sodium chloride in 0.1 N concentration is added, no change in colour or spectrum occurs (Fig. 2, *p*). If eosin in the form of free acid is employed the colour becomes of a lighter and purer violet pink and the absorption bands become lighter, without, however, changing their position (Fig. 2, *r*).

#### OSMOTIC PRESSURE

Further data are necessary to elucidate the nature of the staining act, which forms the subject of this investigation. In particular it is desirable to ascertain the osmotic pressure exerted by the dyes. Observations of this kind made with aniline dyes appear to be limited to some determinations made by Krafft,<sup>1</sup> who found that the molecular weight of perfectly dry rosaniline chloride, methylenblue and methylviolet, ascertained by observing the raising of the boiling-point of absolute ethyl alcohol, corresponded to the accepted formulae of these dyes, but that when care was not taken to exclude the presence of traces of water, or when water was used as the solvent, the raising of the boiling-point was less than that required by theory and an apparent molecular weight, perhaps twice as large, was obtained. The latter observation is of considerable significance, for, taken in conjunction with the well known property which such dyes exhibit to separate out from their solvents not in the crystalline, but in the amorphous or globomorphous state, it indicates that these substances are in the latter case present in the colloidal form. Whether the dye is present in an imperfectly developed colloidal state still capable of exerting a definite osmotic pressure, or whether it is present in two different states, being partly in colloidal, and partly in true, solution, is not yet determined, but the latter view is probably correct since it is found that, when the dye is added in successive amounts to the solvent, the raising of the boiling point or lowering of the freezing point is most marked after the first addition, and each subsequent addition of the dye is attended with less effect than the preceding

1. 'Ueber colloidale Salze als Membranbildner beim Färbeprocess,' *Ber. d. deut. chem. Gesellschaft*, 1899, 32 Jahrg., Bd. II, S. 1608.

It is difficult to explain this circumstance except on the assumption that the first portion added passes largely into true solution, the solvent becoming rapidly saturated by the quantities of the dye subsequently added, so that the only form in which further solution can take place is the colloidal form. It is interesting to note that Kraft found that fuchsin, methylenblue and methylviolet diffused through parchment paper, while benzopurpurin, benzazurin and azoblue showed no trace of diffusion.

In Tables 3 and 4 some determinations<sup>1</sup> are given of the influence of methylenblue-eosin, methylenblue and alcohol soluble eosin upon the boiling point of methyl alcohol, and that of methylenblue and water soluble eosin upon the freezing point of water. In these determinations no attempt was made to obtain the dyes in a perfectly dry condition,<sup>2</sup> since it was desired to investigate their condition under circumstances similar to those obtaining in the staining liquids, alcoholic and watery, used in histological technique, in the preparation of which no attempt is made to exclude the presence of traces of water from the dyes employed.

The results obtained in Tables 3 and 4 show that methylenblue-eosin, as also methylenblue and both forms of eosin, dissolved in methyl alcohol or water, exist in the colloidal state, thus resembling fuchsin, methylviolet, tannin and soap. The last column of these Tables gives the amount of dye which may be regarded as present in true solution, calculated on the assumption that the dyes exist in a diphasic condition. These results are, however, in all probability, except in the case of Exp. 6, Table 3, too high owing to the difficulty of completely removing from the dyes slight impurities, which may exert osmotic pressure. The disturbing influence of even mere traces of such impurities is readily understood, when it is borne in mind that a 0.01 N concentration of methylenblue-eosin, for example,

1. With Beckmann's apparatus, *Zeitschr. f. physikal. Chemie*, 1902, Bd. XL, S. 129.

2. The dyes employed contained 7% to 12% of water. If methylenblue-eosin is dried to constant weight at 100° C., it becomes very slightly soluble in alcohol, requiring repeated renewal of the solvent in order to obtain complete solution; the spectrum of the dye is not altered. The same is true of alcohol soluble eosin. In neither case is the solubility improved by soaking the dried dye again in water. On the other hand pastilles of the freshly precipitated dye, incompletely dried in air, dissolve at once in methyl alcohol.

would be represented by a 1·2% solution, while the same concentration of sodium chloride would be represented by a '06% solution. Alcohol soluble eosin can readily be obtained in a pure condition by adding an equivalent amount of sulphuric acid to water soluble eosin in aqueous solution ; Exp. 6, Table 3, was made with eosin so precipitated,<sup>1</sup> and subsequently thoroughly washed and incompletely dried in air, while Exp. 5, Table 3, was made with a sample obtained from Grübner. The purification of the remaining dyes, in particular the separation of small quantities of salt, is attended with great difficulty. Sodium chloride, for example, is formed when methylenblue and water soluble eosin are mixed in the required proportion ; if the bulky precipitate thus formed is washed with large quantities of distilled water serious loss from solution of the dye, which is highly insoluble only in saline liquids, occurs. In Exp. 3, Table 4, a sample of eosin puriss.<sup>2</sup> was employed. In Exp. 4, the dye was prepared by dissolving freshly precipitated and carefully washed alcohol soluble eosin in the calculated amount of a solution of pure sodium hydrate and evaporating to dryness<sup>3</sup> ; a lower degree of osmotic pressure was noted. The methylenblue employed was supplied by Grübner in a specially purified form suitable for injection *intra vitam*. The sparing solubility of methylenblue-eosin in alcohol renders difficult accurate ebullioscopic observations, the degree of alteration of the boiling point obtained being inconveniently small ; with water as solvent no such observations are possible.<sup>4</sup>

The difficulty of ensuring the absence of traces of electrolytes from the dyes investigated renders determinations of the conductivity of these solutions of little value in affording knowledge of the degree of ionisation present.

1. For analysis, see p. 409.

2. For analyses of these dyes see pp. 407-411.

3. Commercial water soluble eosin may exhibit more than twice the osmotic pressure required by theory for the pure salt.

4. It is interesting to note that Michaelis (Dent. medizin. Wochenschr., 1904, No. 42; Virchow's Archiv, 1905, Bd. 179, S. 195) finds that watery solutions of eosin and methylenblue, though optically inhomogeneous, are not resolvable into submicroscopic particles. Michaelis and also Zsigmondy (Zur Erkenntniß der Kolloide, 1905, S. 160) found, however, that a watery solution of suchsin was partially resolvable into ultramicroscopic particles, whence both observers conclude that this dye is diphasic in such solution.

Although the osmotic pressure of methylenblue-eosin in aqueous solution cannot be determined, the marked tendency of such solutions to form films and amorphous precipitates of the dye, especially on the addition of a trace of neutral salt, such as sodium chloride, shows the highly colloidal nature of such solutions. Alcohol soluble eosin in aqueous solution can be equally readily precipitated by neutral salts ; methylenblue is similarly precipitated, but stronger concentration of salt is required. Water soluble eosin is not precipitated by sodium chloride or iodide.

It appears, therefore, that all the stains dealt with in this paper exhibit colloidal characters in alcoholic and aqueous solutions.

The occurrence of the colloidal state probably explains why it is that no recognisable degree of hydrolysis could be recognised by the method of observation adopted in the preceding section. It would appear that in the colloidal state the dyes in question are shielded from the dissociative influence of the solvent.

#### SUMMARY

The main conclusions resulting from the present investigation are the following :—

1. The staining act of methylenblue-eosin in alcoholic solution is a chemical reaction.
2. Methylenblue-eosin in alcoholic and aqueous solution exhibits colloidal characters, as do also methylenblue and eosin in the water soluble and alcohol soluble forms.

## THE STAINING ACT

TABLE I. COLOUR AND FLUORESCENCE OF DYES EMPLOYED

In the proportion of six molecules of potassium hydrate to one molecule of methylenblue. In '001N concentration, alcohol soluble eosin is of a darker red colour than water soluble eosin in the same concentration.

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## MINIMIZATION OF THE AMOUNTS OF METHYLENBLUE AND OF EOSIN REQUIRED EQUALITY OF COLOUR WITH A SOLUTION OF METHYLENBLUE-EOSIN

No. of exp.	Methylene-blue+eosin	CONCENTRATION OF		SOLVENT	Mode of OBSERVATION	RESULT	REMARKS
		Methyleneblue	Eosin				
1	$56 \times 10^{-5}$ N	$54 \times 10^{-5}$ N	Water sol. $48 \times 10^{-5}$ N	Alcohol	{ Methyleneblue+eosin placed in one test-tube; methyleneblue and eosin put in a second test-tube of the same diameter as the first }	Complete equality of colour	Cp. spectra A, B and D, fig. 1 <sup>1</sup>
2	"	" 53 "	Alc. sol. " 50 "	"	{ Methyleneblue+eosin, methyleneblue and eosin placed separately in glass cells, the two latter being apposed and comparison made with the first }	Very nearly complete equality of colour	Cp. spectrum C, fig. 1 <sup>1</sup>
3	"	" 54 "	Water sol. " 48 "	"	{ Methyleneblue+eosin placed in one test-tube, methyleneblue and eosin put in a second test-tube of the same diameter as the first }	Complete equality of colour	Cp. spectra E, F and H, fig. 1 <sup>1</sup>
4	"	" 53 "	Alc. sol. " 50 "	"	{ Methyleneblue+eosin placed in one test-tube, methyleneblue and eosin put in a second test-tube of the same diameter as the first }	Very nearly complete equality of colour	Cp. spectrum G, fig. 1 <sup>1</sup>
5	"	" 54 "	Water sol. " 48 "	Water	In test-tubes, as in 1 and 2	Complete equality of colour	
6	"	" 53 "	Alc. sol. " 60 "	"	"	Very nearly complete equality of colour immediately after mixing	
7	"	" 54 "	Water sol. " 48 "	"	In glass cells, as in 3 and 4	Methyleneblue+eosin: too blue	Nearly complete equality of colour
8	"	" 54 "	" 82 "	"	"	Methyleneblue+eosin: too blue	Nearly complete equality of colour
9	"	" 53 "	Alc. sol. " 50 "	"	"		
10	"	" 53 "	" 77 "	"	"		

The concentration employed for the spectra given in fig. 1 is five times that employed in this table.

## THE STAINING ACT

TABLE III. RAISING OF THE BOILING POINT OF METHYL ALCOHOL, BROUGHT ABOUT BY  
METHYLENBLUE-EOSIN, METHYLENBLUE AND ALCOHOL SOLUBLE EOSIN. AMOUNT  
OF SOLVENT EMPLOYED 15·78 g. (MOLECULAR RAISING OF B.P. = 8·8° C.).

No. of Experiment	Dye	Amount of Dye employed	Molecular Concentration of Dye	Observed Raising of Boiling Point	Corresponding Molecular Concentration (calculated)	Observed Raising of Boiling Point in Percentage of Calculated Amount
1	Methylenblue-eosin, 1st sample...	0·098 g.	0·0052 N.	+ 0·010° C.	0·0011 N.	22
2	" " 2nd , , ,	0·100 g.	0·0053 N.	+ 0·030° C.	0·0037 N.	71
3	Methylenblue ... , , ,	0·353 g.	0·0283 N.	+ 0·091° C.	0·0104 N.	37
4	" " , , ,	0·101 g.	0·0081 N.	+ 0·013° C.	0·0015 N.	18
5	Eosin (alcohol soluble) 1st sample	0·154 g.	0·0152 N.	+ 0·016° C.	0·0018 N.	12
6	" " , , 2nd , ,	0·146 g.	0·0144 N.	+ 0·000° C.	—	0

TABLE IV. LOWERING OF THE FREEZING POINT OF WATER, BROUGHT ABOUT BY METHYLENBLUE  
AND WATER SOLUBLE EOSIN (SODIUM SALT). AMOUNT OF SOLVENT EMPLOYED 13 g.  
(MOLECULAR LOWERING OF F.P. = 19° C.).

No. of Experiment	Dye	Amount of Dye employed	Molecular Concentration of Dye	Observed Lowering of Freezing Point	Corresponding Molecular Concentration (calculated)	Observed Lowering of Freezing Point in Percentage of Calculated Amount
1	Methylenblue ... , , ,	0·358 g.	0·035 N.	- 0·102° C.	0·0054 N.	15
2	" " , , ,	0·031 g.	0·003 N.	- 0·010° C.	0·0005 N.	19
3	Eosin (water soluble) 1st sample	0·447 g.	0·010 N.	- 0·781° C.	0·0412 N.	82
4	" " , , 2nd , ,	0·143 g.	0·016 N.	- 0·106° C.	0·0050 N.	31

## SECRETIN IN RELATION TO DIABETES MELLITUS

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*(Received July 30th, 1906)*

### SECTION I

#### THE TREATMENT OF DIABETES WITH SECRETIN

The relationship between the pancreas and diabetes has long been recognised, and it has been supposed that many cases of diabetes result from the deficiency or absence of an internal secretion normally produced by the pancreas. Starling suggested that secretin might furnish the stimulus not only to the external but also to this internal secretion, and that it might prove to be of value in the treatment of diabetes. But Spriggs, who injected intravenously once a day 5 c.c. of a secretin solution freed from depressor substance, obtained a negative result in a patient who died of coma a week after the treatment had been begun.

Recently Moore, working with Edie and Abram, has published the results obtained by giving secretin by the mouth to three patients suffering from diabetes. Their first case—a man aged 25—at the beginning of the treatment was passing 40-65 grms. of sugar daily on a diabetic diet with phenacetin. He then received 30 c.c. of secretin solution three times daily. After a latent period of three weeks the sugar fell suddenly to 32 grms., and for several weeks varied from 21-30 grms. daily. After four months' treatment with secretin the urine became free from sugar, and the patient gained in weight. The patient returned to work, and the secretin treatment was discontinued. Six months later he returned to the Hospital suffering from phthisis. His urine contained about 120 grms. of sugar daily ; secretin was again administered, but without effect. The other two

cases were children aged 7 and 9 respectively. In the first, the secretin treatment was begun simultaneously with the administration of a rigid diabetic diet ; the quantity of sugar in the urine gradually fell, and the urine became sugar free at the end of forty-six days. The second child was put upon a diabetic diet together with small quantities of carbohydrate ; twelve days later treatment with secretin was begun and in twenty-four days the urine was sugar free. Moore suggests that the extract of duodenal mucous membrane supplied to the pancreas a chemical excitant, which was lacking in the diabetic patients ; this caused the pancreas to resume its formation of internal secretion, and as a result the patients regained the power of metabolising carbohydrate, and retained this power for some time after the omission of the secretin but not of the strict diet.

In all of these cases the treatment by secretin was combined with a diabetic diet more or less rigid. It is necessary, therefore, to consider whether results similar to those observed by Moore are seen in similar cases of diabetes treated by dieting alone. Clinically, diabetes may be divided into two grades of severity, mild and severe. In the first, the dextrose in the urine appears to come only from the carbohydrate of the food. When such a patient is placed on a rigid diabetic diet, his urine may be expected to become sugar free within a week, and not to give a reaction with ferric chloride ; the patient rapidly improves and may regain a very considerable power of utilising carbohydrate food. The other type of the disease is different clinically, in that dextrose is still passed out, although the patient is having the most rigid diet which can be devised. In the most severe cases the patient, when on an ordinary diet, passes out much sugar and diacetic acid ; when placed on a rigid diet the sugar in the urine decreases for a time, but the diacetic acid and acid intoxication rapidly increase, so that within a fortnight the patient may suffer from nausea, loss of appetite and other symptoms of threatened coma, from which he may or may not spontaneously recover. However long the strict diet is maintained, the sugar in the urine never disappears, and very often it tends to rise after the initial fall. None of Moore's cases were of such severity as this. The mildest cases of this type give a very

different response to treatment by diet. Such patients, when on an ordinary diet, may or may not pass out diacetic acid besides sugar ; when placed on a rigid diet, diacetic acid is passed and the sugar after fluctuations decreases, so that the urine may become sugar free in a month or two. Considerable power of utilising carbohydrate may be gained, the diacetic acid reaction may disappear, and the patient may pass for a time into the mild type of diabetes. From the histories of Moore's cases, and from the analyses of the urines given by Edie and Whitley, it would appear that Cases II and III were of this nature, and that of the two, Case II was rather the more severe. It is a well known fact that cases rather more severe than either of these may become sugar free after a long period of dieting, but that if they go back to ordinary diet for several months and then come under treatment again, it may be found that the type of disease has undergone a considerable change for the worse ; this appears to have happened in Moore's first case. In fact there is no evidence to show that any of Moore's cases were of such severity that dieting alone would not have accounted for the observed disappearance of sugar from the urine. It seemed desirable, therefore, to test the effect of secretin on diabetics of a rather more severe type.

*Methods.*—The extracts used were obtained from the intestinal mucous membrane of pigs, sheep, and other animals. The upper two or three feet of the intestine were used, and the extracts were made by the method described by Bayliss and Starling. In a majority of cases the activity of the extracts was tested by experiment on animals and, if need arose, the extracts were concentrated. In the first instance the extract was injected subcutaneously in 10-20 c.c. doses, but the patients objected to the injections as they caused much pain locally and sometimes severe headache ; consequently, after one or two injections the secretin was given only by mouth. Each patient received 30 c.c. two or three times daily, one hour after food. The total urine for the 24 hours was collected and measured each day ; the sugar was estimated by Pavvy's method, and the ferric chloride test for diacetic acid was made use of.

## DESCRIPTION OF CASES

We have treated three cases of the severe type of diabetes with secretin. Case I was clinically the least severe; Case III was suffering from diabetes in its most severe form, and Case II was rather less severe than this.

*Case I.*—A boy, aged 15, was admitted into Guy's Hospital on January 31st, 1906, for thirst and wasting. These symptoms had been noticed only since the previous December.

On admission the patient was found to be extremely thin; his urine contained a large amount of sugar, but neither acetone nor diacetic acid. For two days he was placed on an ordinary diet, and on February 2nd, this was changed to a rigid diabetic diet with the daily addition of 35 grams of carbohydrate given at dinner time in the form of potatoes. On February 26th, the daily allowance of carbohydrate was reduced to 25 grams. On February 28th, the urine began to give a reaction with ferric chloride, which disappeared on March 4th, when the urine contained only a trace of sugar. On March 5th, the urine became sugar free after thirty-two days of treatment by dieting. Between February 2nd and March 1st, the patient received 20 grams of sodium bicarbonate daily; this was stopped as the urine became alkaline to litmus on March 1st.

On March 6th, the patient was put back upon the ordinary full hospital diet, and on March 8th, 10 c.c. of secretin solution were injected subcutaneously. Between March 9th and 19th the patient received 30 c.c. of secretin solution by the mouth, either once, twice or three times a day. During this period the urine contained increasing quantities of sugar, but gave no diacetic reaction.

On March 20th, the patient was put on the most rigid diabetic diet which could be devised, it contained no milk, and the substitutes for bread used were Protene bread and biscuits, and Callard's brown caseoid bread. The diacetic reaction in the urine returned at once, and the sugar diminished, but had not disappeared at the end of nine days.

The characters of the urine, the amounts of sugar passed, and other details are given in the following table:—

1906 Date	Daily Amount of Urine in c.c.	Total Sugar in grams	Dose of Secretin in c.c.	Weight of Patient in kilos.	Other Characters of Urine
Jan. 31	1931	106.5*	—	—	Acid, no acetone nor diacetic acid.
Feb. 1	2397	203.6*	—	—	” ” ” ” ”
” 2	Diabetic diet with 35 grams Carbohydrate and 20 grams sodium bicarbonate.				
” 4	1130	113.4*	—	—	Acid, no acetone nor diacetic acid.
” 5	1760	101.9*	—	—	” ” ” ” ”
” 6	1470	91.8*	—	—	” ” ” ” ”
” 10	1470	81.5*	—	—	” ” ” ” ”
” 12	1820	103.7*	—	—	” ” ” ” ”
” 22	1480	47.4*	—	—	” ” ” ” ”
” 26	Carbohydrate reduced to 25 grams.				

1906 Date	Daily Amount of Urine in c.c.	Total Sugar in grams	Dose of Secretin in c.c.	Weight of Patient in kilos.	Other Characters of Urine
Feb. 28	2470	99.8	—	—	Acid, faint diacetic reaction.
Mar. 1	2850	105.8	—	—	Alkaline     "     "
2	1550	53.1	—	—	Neutral, weak     "     "
3	890	14.8	—	34.5	Acid,     "     "     "
4	1220	Trace	—	—	"     no diacetic reaction.
5	1220	Absent	—	34.9	"     "     "
March 6th, the patient was given ordinary diet.					
6	2400	89.1	—	—	Acid, no diacetic reaction.
7	2360	147.5	—	33.8	"     "     "
8	2410	180.7	10 (subcut.)—	—	"     "     "
9	2620	218.9	30 (mouth)	33.8	"     "     "
10	2540	205.7	30	—	"     "     "
11	3180	242.2	60	34.1	"     "     "
12	2660	216.5	60	—	"     "     "
13	2950	247.4	90	34.6	"     "     "
14	3530	273.3	90	—	"     "     "
15	2970	266.5	90	34.9	"     "     "
16	3130	288.2	60	—	"     "     "
17	2470	233.5	60	35.0	"     "     "
18	3280	330.6	60	—	"     "     "
19	2500	233.2	90	35.2	"     "     "
March 20th, patient was put on a rigid diabetic diet.					
20	1330	58.5	—	—	Acid, trace diacetic acid.
21	1370	17.8	—	—	"     "     "     "
22	1250	36.3	—	—	"     "     "     "
23	1510	45.8	—	—	"     "     "     "
24	1440	32.4	—	—	"     "     "     "
25	1610	54.0	—	—	"     "     "     "
26	1440	54.0	—	—	"     "     "     "
27	1230	34.5	—	—	"     weak diacetic reaction
28	1930	68.9	—	—	"     "     "     "

Estimations marked \* were made by the Ward Clerk before the case came into our hands.

After leaving the hospital the boy went back to an ordinary diet for several months. Recently he has been under the observation of one of us; his urine contains much sugar and diacetic acid, and can no longer be made sugar free by a restricted diet.

*Case II.*—A man aged 49, was admitted into Guy's Hospital on March 3rd, 1906, for cough and shortness of breath. He had been in the hospital previously and was known to have suffered from phthisis and diabetes since December, 1903.

On admission the patient was found to be thin, he was suffering from chronic fibroid phthisis, and throughout his stay in the hospital showed no pyrexia; his urine contained

a moderate amount of sugar, gave no diacetic acid reaction and did not contain albumen. He was placed at once upon a rigid diabetic diet, together with 35 grams carbohydrate given daily at dinner time in the form of potatoes. The same diet was continued during and subsequent to the period of treatment with secretin.

On March 6th, the urine began to give a reaction with ferric chloride which increased in intensity until, on March 12th and 13th, it was a strong reaction ; from this date the intensity decreased, and on March 21st, the reaction was only weak. On March 7th, the patient felt sick and lost his appetite ; these symptoms of incipient coma lasted for two days and did not recur.

On March 6th, 10 c.c. of secretin solution were injected subcutaneously and on March 8th and 9th, 20 c.c. These injections gave the patient not only a severe headache, lasting several hours, but the last produced such a severe fall in the blood pressure as to be dangerous.

The output of sugar in the urine showed no tendency to diminish in amount during or after the period of treatment with secretin ; on the whole it rose slowly and steadily throughout the period of observation.

The progress of the case is shown in the following table :—

1906 Date	Daily Amount of Urine in c.c.	Total Sugar in grams	Dose of Secretin in c.c.	Weight of Patient in kilos.	Other Characters of Urine
Mar. 3	950	31.2	—	—	No diacetic reaction.
„ 4	1420	45.3	—	—	„ „ „
„ 5	1570	52.3	—	—	Trace „ „
„ 6	1700	54.9	10 (subcut.)	—	Moderate diacetic reaction.
„ 7	1830	63.2	—	—	„ „ „
„ 8	1900	65.1	20 (subcut.)	54.7	„ „ „
„ 9	2160	79.0	20 (subcut.)	—	„ „ „
„ 10	1670	62.2	30 (Mouth)	54.2	„ „ „
„ 11	1430	63.8	60	—	„ „ „
„ 12	1970	75.2	60	55.2	Strong „ „
„ 13	1770	70.8	90	—	„ „ „
„ 14	1810	88.7	90	54.2	Moderate „ „
„ 15	1850	89.8	90	—	„ „ „
„ 16	1730	85.7	60	54.3	„ „ „
„ 17	1630	81.8	60	—	„ „ „
„ 18	1490	86.2	60	54.3	Weak „ „
„ 19	1630	85.3	90	55.0	„ „ „
„ 20	1420	76.8	90	—	„ „ „
„ 21	1920	86.5	90	54.9	„ „ „
„ 22	2110	97.7	90	—	„ „ „
„ 23	1650	85.9	90	54.8	„ „ „
„ 24	1560	83.7	90	—	„ „ „
„ 25	1270	70.2	—	54.9	„ „ „
„ 26	1340	78.3	—	—	„ „ „
„ 27	1260	71.3	—	55.9	„ „ „

*Case III.*—A man, aged 45, was admitted into Guy's Hospital on March 1st, 1906, for thirst, polyuria and wasting. The patient had been losing weight for about a year and had noticed polyuria for six months. His daughter, aged 8, had died in the Hospital of diabetic coma just previously to his own admission. Her case is referred to as *Case I* in Section II of this paper.

On admission the patient was found to be very wasted; his breath smelt strongly of acetone; his urine contained diacetic acid, acetone and a moderate quantity of sugar. He was placed at once on a rigid diabetic diet, together with 35 grams carbohydrate given as potatoes. The same diet was maintained until the end of the period of observation.

On March 2nd, the urine gave a moderate reaction with ferric chloride; this increased to a strong reaction on March 6th, and continued so until the end. On March 7th, the patient complained of nausea, could eat very little and felt too ill to get up. The next day he vomited, ate practically no food, and was stupid and rather drowsy. On March 9th, the patient ate 4 ounces of ordinary bread and some milk; by the next day the symptoms of incipient coma had begun to disappear and the patient went back to his proper diet. On March 8th, the patient was given 15 grams of sodium bicarbonate; the dose was increased to 30 grams on the next day, and this quantity was given daily until March 19th, when the urine became alkaline to litmus; after this the drug was discontinued.

On March 6th, 10 c.c. of secretin solution were injected subcutaneously, and on 8th, 20 c.c. After that date 30 c.c. were given once, twice, or three times daily by the mouth until 24th. During the first ten days of the treatment by secretin the amount of sugar in the urine varied but little, although on the whole it tended to increase; but on March 19th, the output became suddenly greater and continued at this higher level during the rest of the secretin treatment and after it was stopped. The striking alterations in weight were undoubtedly due to retention of water, following the administration of sodium bicarbonate; after March 16th, the daily quantity of urine rose and the stored up fluid was got rid of.

The following table shows the progress of the case:—

1906 Date	Daily Amount of Urine in c.c.	Total Sugar in grams	Dose of Secretin in c.c.	Weight of Patient in kilos.	Other Characters of Urine
Mar. 2	2160	79.0	—	—	Acid, moderate diacetic reaction
„ 3	2010	75.4	—	46.7	„ „ „ „
„ 4	2150	81.0	—	—	„ „ „ „
„ 5	2430	83.4	—	47.2	„ „ „ „
„ 6	2820	67.8	10 (subcut.)	—	„ strong „ „
„ 7	3290	86.1	—	47.2	„ „ „ „
„ 8	2470	71.0	20 (subcut.)	—	„ „ „ „
„ 9	2240	91.0	30 (mouth)	46.5	„ „ „ „
„ 10	2150	96.9	30	—	„ „ „ „
„ 11	2250	94.5	60	48.5	„ „ „ „
„ 12	2580	97.0	60	—	„ „ „ „

1906 Date	Daily Amount of Urine in c.c.	Total Sugar in grams	Loss of Secretin in c.c.	Weight of Patient in kilos.	Other Characters of Urine			
					Acid, strong	diacetic	reaction	"
Mar. 13	2470	95.4	90	51.0				
" 14	2350	89.4	90	—	"	"	"	"
" 15	2620	102.3	90	51.4	"	"	"	"
" 16	3330	120.6	60	—	"	"	"	"
" 17	3590	111.7	60	49.8	"	"	"	"
" 18	3600	108.4	60	—	Neutral	"	"	"
" 19	4300	152.3	90	47.7	Alkaline	"	"	"
" 20	3680	134.3	90	—	Acid	"	"	"
" 21	4210	169.8	90	46.8	"	"	"	"
" 22	4580	183.2	90	—	"	"	"	"
" 23	4600	184.7	90	47.1	"	"	"	"
" 24	4130	163.3	90	—	"	"	"	"
" 25	3950	145.2	—	46.8	"	"	"	"
" 26	4380	158.8	—	—	"	"	"	"
" 27	4050	154.6	—	—	"	"	"	"

### CONCLUSIONS

It will be seen from the preceding account that in three cases of the severe type of diabetes, the administration of secretin by the mouth had no influence whatever upon the output of sugar in the urine. And yet it was possible in *Case I*, after a latent period of more than a month, to abolish the glycosuria by means of dieting. It seems probable that Moore's cases were of a kind similar to this one, and that the long latent period, which he attributed to secretin, belonged to the diet. There appears to us to be no sufficient evidence that secretin, when given by the mouth, can abolish or influence the glycosuria of severe diabetes.

In the light of Starling's experience, that in animals secretin is not absorbed when introduced even in large amount into the alimentary canal, it seemed desirable to try the effect of full doses of secretin given subcutaneously. The number of subcutaneous injections given by us is too small to enable us to form any opinion of their value in the treatment of diabetes.

## SECTION II.—PROSECRETIN IN DIABETES

The treatment of diabetes mellitus with secretin is based primarily upon the assumption that prosecretin is deficient in, or absent from, the intestinal mucous membrane of either all, or at least some, diabetic patients. It seemed desirable to ascertain whether this assumption was justified by the facts. We have, therefore, examined for prosecretin the intestines of six diabetic patients. As a control, we have compared the results so obtained with those given by the intestines taken from nine patients dying of other diseases.

## METHODS

The duodenum and upper part of the small intestine was obtained from diabetic and other patients as soon as possible after death ; the mucous membrane was scraped off, and an acid extract made according to the method described by Bayliss and Starling. The activity of the extract was tested by intravenous injection into cats or dogs, anaesthetised with ether or A. C. E. mixture, and having a cannula in the pancreatic duct. In every experiment, the effect of the extract from diabetic mucous membrane was compared with that obtained by injecting secretin made either from normal animals or from non-diabetic patients. Many of the diabetic extracts were tested twice in different animals. As a rule, the blood pressure was recorded in the experimental animals, and the depressor effect observed. Further details appear in the protocols.

## RESULTS

*A. Non-Diabetic Patients.*—It was necessary, in the first place, to determine whether prosecretin disappeared shortly after death or whether its presence could always be demonstrated in the duodenal mucous membrane, even one or two days post mortem. The duodenum was taken from the bodies of nine patients at varying intervals after death ; it yielded secretin in every case. The results of these control experiments are indicated in the following table :—

CASE	SEX, CAUSE OF DEATH	Interval after death at which material was obtained and extracted	Secretion of Juice obtained by Injection
I.	Boy. Fibroid phthisis	46 hours	Fair.
II.	Woman. Uraemic coma	30 "	Fair.
III.	Man. Granular kidneys	25 "	Moderate.
IV.	Woman. Mitral Disease and phthisis	24 "	Abundant.
V.	Boy. Tuberculous Menin- gitis and general tubercu- losis	11 "	Fair.
VI.	Man. Cirrhosis of liver	7 "	Fair.
VII.	Child. Mastoid abscess and thrombosis of lateral sinus	7 "	Abundant.
VIII.	Man. Pneumonia	6 "	Abundant.
IX.	Woman. Carcinoma of pylorus. Gastro-jejunos- tomy; peritonitis	4 "	Fair.

From this table it is obvious that, although it is possible that there is some post-mortem disappearance of prosecretin, the duodenal mucous membrane contains a fairly large quantity of it as long as two days after death.

The following protocol, which will serve as an example, shows the presence of a normal quantity of prosecretin 24 hours after death :—

*Experiment IV.*—Woman, aged 20, died from mitral disease and phthisis. Duodenum obtained 24 hours after death; extract made at once in the usual way.

Injected 2 c.c. extract into cat anaesthetised with ether. Blood pressure fell from 130-85 mm. Hg. Secretion of juice 0.5 c.c. Subsequent injections yielded a similar secretion of juice.

*B. Diabetic Patients.*—We have had the opportunity of examining the duodenum of six diabetic patients for the presence of

prosecretin ; three died of diabetic coma, and three died of intercurrent disease. In view of the great importance of the clinical history in determining the grade and severity of the diabetes, we have given an account of each of these cases, in addition to the following general summary of the results obtained.

CASE	SEX, AGE, AND CAUSE OF DEATH	Clinical Duration of Disease	Interval between Death and Prepara- tion of Secretin	Flow of Juice produced by Extract
I.	Girl, aged 8. Diabetic coma ... ... ...	1 month	14 hours	Nil.
II.	Man, aged 27. Diabetic coma ... ... ...	22 months	14 , ,	1 drop from a maximal dose.
III.	Man, aged 30. Diabetic coma ... ... ...	10 , ,	25 , ,	Nil.
IV.	Man, aged 48. Diabetes and phthisis ... ...	18 , ,	40 , ,	Very scanty.
V.	Woman, aged 58. Dia- betes, coma and pye- lonephritis ... ...	5 , ,	48 , ,	Moderate.
VI.	Man, aged 67. Diabetes. Septicaemia ... ...	4 years	46 , ,	Very scanty.

*Case I.*—Girl, aged 8, was admitted into Guy's Hospital on February 21st, 1906, for wasting and thirst. Her father was subsequently admitted into the Hospital suffering from severe diabetes, and is referred to as *Case I* in Section I of this paper. She had been noticed to be getting thinner for five weeks and had complained of thirst for three weeks. On admission she was found to be extremely thin ; she was rather drowsy and showed slight 'air-hunger' ; her urine contained a trace of albumen, acetone, but no diacetic acid, 2.5% sugar ; she passed 6.5 litres of urine in the first 24 hours. On February 25th, she became definitely comatose and died the next day. The post-mortem took place 17 hours after death ; all the organs were found to be healthy, excepting that some of the mesenteric glands were large and caseous. The pancreas was soft and weighed 18 grams ; microscopically it was normal.

The duodenum was received and extracted 14 hours after death. The extract was tested upon a cat anaesthetised with ether, and compared with extracts from non-diabetic *Case IV* and normal cat's intestines. The results are recorded in the following protocol :—

INJECTION		BLOOD PRESSURE	FLOW OF JUICE
4 c.c. extract from diabetic case I	...	130 fell to 80	Nil.
2 c.c. " " non-diabetic case IV.	...	130 " " 85	0.5 c.c.
5 c.c. " " diabetic case I	...	110 " " 70	Nil.
5 c.c. " " " "	...	110 " " 60	Nil.
2 c.c. " " non-diabetic case IV	...	110 " " 70	0.5 c.c.
4 c.c. " " diabetic case I	...	110 " " 70	Nil.
5 c.c. " " normal cat	...	110 " " 65	0.5 c.c.

*Case II.*—A man, aged 27, was originally admitted into Guy's Hospital on September 16th, 1904, for weakness, thirst and polyuria of two months' duration. On admission he was found to be thin ; his urine measured about six litres and contained 5% sugar, acetone and diacetic acid. He was dieted and his urine became sugar-free on November 30th, and at the same time the diacetic reaction disappeared. The man left the hospital on February 16th, 1905.

He was readmitted on May 18th, 1906, for congenital lamellar cataract. He was passing daily about 20-30 litres of urine containing about 1.5% dextrose. On June 9th, he developed 'air-hunger' and died, comatose, on the next day. The post-mortem examination showed that the body was much wasted, and that all the viscera were normal excepting for some old pleural adhesions ; the pancreas weighed 54 grams, and was microscopically normal.

The intestines were received 14 hours after death and an acid extract was made at once. The extract was tested upon a cat anaesthetised with ether, and compared with extracts made from the intestines of a normal dog and of a cat fourteen days after removal of the pancreas. The following protocol gives the results of the experiment :—

INJECTION		BLOOD PRESSURE	FLOW OF JUICE
2 c.c. extract from depancreatized cat	...	150 fell to 70	0.5 c.c.
3 c.c. " " patient	...	140 " " 60	1 drop
2.5 c.c. " " depancreatized cat	...	110 " " 75	0.3 c.c.
2.5 c.c. " " normal dog	...	145 " " 60	0.7 c.c.
5 c.c. " " depancreatized cat	...	120 " " 50	1.0 c.c.

The extract from this patient was tested thoroughly on two other animals and gave generally no flow of juice at all, or in a few instances one drop, even when injected in doses of 10 c.c.

*Case III.*—Man, aged 30 years, was admitted into St. Bartholomew's Hospital for wasting and polyuria. He had been well until ten months previously, since when he had lost two stone in weight and had passed large quantities of urine. While in the hospital he passed daily 3-5 litres of urine containing 100-200 grams of sugar ; the urine contained diacetic acid. Eight days after admission the patient became comatose, and died 24 hours later. *Post-mortem.*—All the viscera appeared to be healthy, and showed very little post-

mortem change. The pancreas weighed 65 grams; it was small and soft, and microscopically normal, excepting perhaps that the section showed a slight excess of fibrous tissue round the small ducts in the gland.

The duodenum was obtained 25 hours after death, and an acid extract of the mucous membrane was made at once. A cat was anaesthetised with ether, and cannulae were placed in the pancreatic duct and one jugular vein. The effects of injecting the extract from the patient's duodenum were compared with those yielded by normal dog's secretin, and are recorded in the following protocol :—

TIME	INJECTION	FLOW OF JUICE
2-55 p.m.	Injected 3 c.c. extract from patient	... Nil.
2-58 "	3 c.c. normal dog's secretin	... 3 c.c.
3-31 "	3 c.c. extract from patient	... Nil.
4-3 "	3 c.c. " " "	... Nil.
4-28 "	4 c.c. " " "	... Nil.
4-35 "	3 c.c. normal dog's secretin	... 0.8 c.c.

The extract from the patient was concentrated and contained much depressor substance.

*Case IV.*—Man, aged 48, was admitted into St. George's Hospital, under Dr. Ogle, on April 23rd, 1906, for phthisis and diabetes. His illness had begun gradually 18 months previously with cough, loss of appetite, progressive wasting and thirst ; he did not notice polyuria. On admission he was found to be extremely ill ; the sputum contained tubercle bacilli ; his legs were oedematous up to the thighs. His urine contained albumen, 8% dextrose, but not acetone. He was placed on a milk diet. The patient gradually became weaker and repeatedly vomited black blood. On April 28th, he died from cardiac failure, not from diabetic coma.

The post-mortem examination was made 12 hours after death. Both lungs showed extensive active tuberculosis. The heart was enlarged on its right side. The liver weighed 53 ozs.; it was fatty and nutmeg. The pancreas weighed 113 grains; it was normal microscopically. The stomach and intestines contained altered blood; no source of haemorrhage could be found.

The intestines were obtained 16 hours after death and were kept in ice for another 24 hours. An acid extract was then made in the usual way and tested on a cat anaesthetised with ether. The results were compared with that yielded by normal dog's secretin, as is shown in the following protocol:—

NATURE OF INJECTION		BLOOD PRESSURE	FLOW OF JUICE
7 c.c. extract from patient	...	Fell from 155 to 75 mm. Hg.	Nil.
6 c.c. normal dog's secretin	...	„ „ 150 „ 80 „ „	1 c.c.
8 c.c. extract from patient	...	„ „ 135 „ 70 „ „	2 drops
10 c.c. „ „ „	...	„ „ 130 „ 70 „ „	2 drops

*Case V.*—Woman, aged 58, was admitted into St. Thomas's Hospital on May 4th, 1906, under the care of Dr. Acland, for diabetes. She had suffered from polyuria and thirst for 5 months, and had noticed loss of weight for 3 months. On admission, the patient was found to be well nourished. Her urine contained a trace of albumen, 3% sugar, and had sp. gr. 1036. She was placed upon a diet in which the carbohydrates were slightly restricted. On May 9th the patient became rather drowsy ; the urine contained traces of diacetic acid and acetone. On the next day she was more drowsy, and the urine contained more diacetic acid and acetone. On May 11th she died in a comatose condition. Her temperature was sub-normal throughout. At the post-mortem examination all the viscera were found to be healthy, excepting the kidneys, which contained a few small abscesses due to pyelonephritis. The pancreas was normal microscopically.

There is a doubt whether the coma in this case was diabetic or uraemic. The opinion of those who observed the case clinically was that the coma was not diabetic.

The intestines were obtained 48 hours after death, having been kept in ice most of the time. The duodenal extract was made in the usual way, and tested upon a dog, anaesthetised with A. C. E. mixture. As a control secretin from a normal dog and from a fatal case of pneumonia (Case VIII of the non-diabetic patients) was injected. In the same dog the duodenal extract of diabetic Case VI was tested, and in the following protocol the results of both cases are given :—

INJECTION	BLOOD PRESSURE			FLOW OF JUICE
9 c.c. extract from non-diabetic Case VIII ...	90	fell to	40 mm. Hg.	2·0 c.c.
8 c.c.     , , diabetic Case V    ...    ...	110	, ,	45    , ,	0·6 c.c.
8 c.c.     , , normal dog ...    ...    ...	120	, ,	40    , ,	1·4 c.c.
9 c.c.     , , diabetic Case VI    ...    ...	90	, ,	40    , ,	1 drop.
8 c.c.     , ,     ,     ,    ...    ...	90	, ,	40    , ,	1 drop.
10 c.c.    , ,     ,     ,    ...    ...	110	, ,	40    , ,	1 drop.
10 c.c.    , ,     ,     ,    ...    ...	90	, ,	40    , ,	1 drop.

*Case VI.*—A man, aged 67, was admitted into Guy's Hospital on May 11th, 1906, in an unconscious condition. Four years previously he had begun to suffer from great thirst and an abnormally large appetite ; one year later he developed cataract in each eye. He had not been feeling well for a week before admission, and on the day of admission he fell down unconscious. When admitted into the Hospital he was found to be well nourished ; his urine contained 1·5% sugar, diacetic acid and acetone, and amounted to 6 litres in the day ; his condition was considered not to be due to diabetic coma. On May 12th he became conscious, but still remained very ill. On May 14th he showed marked orthopnoea, and his dyspnoea was clearly due to cardiac and general circulatory failure. On May 16th his temperature rose to 106° F., and on the next day his left knee joint became inflamed ; he passed about 150-200 grams of sugar daily. On March 18th both knee joints were acutely inflamed, and a pericardial rub was noticed ; the patient became semi-conscious and died on the next day.

The post-mortem examination showed that the man had died of septicaemia and acute septic pericarditis. The heart muscle was fatty, the arteries atheromatous, the liver fatty, and the kidneys tough. The pancreas weighed 80 grams; microscopically it was normal.

The duodenum was received 46 hours after death, and an extract of it was made at once. The effect produced by the injection of the extract into a dog is shown in the protocol at the end of *Case I*.

### CONCLUSIONS

Our observations show that only in one out of six cases of severe diabetes was prosecretin present in an amount approximating to the normal. This one case, however, is sufficient to show that an absence of prosecretin is not a necessary result of the abnormal metabolism of severe diabetes. The same thing is shown by the fact that we found prosecretin present in normal quantity in the duodenum of a cat a fortnight after the removal of the pancreas (*vide* protocol at the end of *Case II*). The cat presented all the symptoms of diabetes in a severe degree; it had lost more than one-third of its original body-weight, the urine gave a reaction with ferric chloride, and the  $\frac{D}{N}$  was about 3.

In the other five cases of severe diabetes, prosecretin was either absent, or present in very small quantities; in the three cases of diabetic coma it was absent, and in two cases of severe diabetes, which died of intercurrent disease, it was present, but extremely scanty. There are two obvious hypotheses which could be advanced to account for the diminution or absence of prosecretin in these cases. In the first place, the acid intoxication, which almost invariably precedes the natural termination in coma, may prevent the normal production of prosecretin; on this view its absence would be a terminal result of the diabetes and would be of very little importance clinically. Secondly, it is possible that prosecretin may be very deficient or absent from the outset in the more acute and severe cases of diabetes, which rapidly progress towards a fatal issue in coma; if this were true, the absence of prosecretin might be the actual cause of such cases of diabetes. But it would be necessary to assume also that secretin is the stimulus to the internal secretion of the pancreas, and that the pancreas is functionally, as well as microscopically, normal.

We have attempted to decide between these two hypotheses by producing an artificial acid-intoxication in animals, and then examining their intestines for the presence of secretin. For this purpose rabbits were used, and dilute phosphoric acid was administered by the alimentary canal; the alkalinity of the blood-serum was estimated by Wright's method and is expressed as the strength of sulphuric acid necessary to neutralise it.

*Experiment.—Rabbit.*

Date 1906	Weight in grams	Alkalinity of Blood-Serum	Quantity of Phosphoric Acid
July 9	4200	$\frac{N}{20}$ (before treatment)	100 c.c. 1%, given per rectum.
„ 10	3970	—	200 c.c. 1%, given per rectum in two doses, one in morning and one in evening.
„ 11	3900	—	100 c.c. 0.5% by mouth and 100 c.c. 1% per rectum. (Animal seemed ill and very feeble).
„ 12	3840	$\frac{N}{50}$	

Rabbit killed. Post-mortem, the stomach and duodenum were normal in appearance. Secretin was prepared from the duodenum in the usual way and its activity tested on a cat, anaesthetised with ether; secretin made from a normal rabbit was used as a control.

INJECTION	FLOW OF JUICE
5 c.c. extract from normal rabbit	... 0.7 c.c.
5 c.c. „ „ injected „	... 0.6 c.c.
5 c.c. „ „ normal „	... 0.5 c.c.
5 c.c. „ „ injected „	... 0.4 c.c.

This experiment shows that prosecretin is not abolished by an acid intoxication which differed not in degree but only in duration, from that observed in cases of severe diabetes. In human diabetes

the duration of the acid-intoxication may extend over months ; and it is possible that such a prolonged acidosis may destroy or inhibit the formation of prosecretin. On the other hand, it is equally possible that a deficiency of prosecretin may be causally related to diabetes. If this were so, it would follow that diabetes should result from an experimental reduction of the prosecretin in the intestines ; and we are attempting to investigate this point. Until more evidence is forthcoming it is impossible to reach a conclusion as to the nature of the relationship existing between prosecretin and some cases of diabetes.

We wish to express our indebtedness to the Physicians of Guy's Hospital, to Dr. Acland, Dr. Ogle, and to Dr. Andrewes of St. Bartholomew's Hospital, for their kindness in supplying us with clinical cases and post-mortem material.

Part of the expense of this investigation was defrayed by a grant from the Royal Society.

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FURTHER OBSERVATIONS ON THE TREATMENT OF  
DIABETES MELLITUS BY ACID EXTRACT OF DUO-  
DENAL MUCOUS MEMBRANE

By BENJAMIN MOORE, M.A., D.Sc., *Johnston Professor of Bio-Chemistry, University of Liverpool*; EDWARD S. EDIE, M.A., B.Sc., *Carnegie Research Scholar*; AND JOHN HILL ABRAM, M.D., F.R.C.P., *Honorary Physician, Royal Infirmary, Liverpool*.

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In a preliminary paper published in this Journal,<sup>1</sup> we described three cases of diabetes mellitus in which improvement appeared to follow administration of an acid extract of duodenal mucous membrane by the mouth, the sugar diminishing gradually and finally disappearing from the urine.

In that paper we were careful to point out, in the first place, that no sweeping conclusions could be drawn from such a small number of cases and that they were given as preliminary in order to excite further work upon the subject; and secondly, that even granting the hypothesis upon which the treatment was based, it was not likely that more than a certain percentage of cases would be benefited by the extract.

The following is a quotation from our previous paper showing the line of argument employed :—

‘If, for the purpose of argument, we take it that the duodenum does yield a chemical excitant for the internal secretion of the pancreas, and that in the absence of the internal secretion glycosuria results, then there are three places in the chain at which weakness due to functional or other disarrangement may occur and lead to a breakdown and the appearance of diabetic conditions. First, the breakdown may occur at the duodenum, on account of the non-secretion of the excitant; secondly, the

1. Vol. I, p. 28.

breakdown may take place at the pancreas, so that although the excitant is formed at the duodenum and carried to the pancreas, yet these cells are not capable of excitation, either from complete morbid change or from some functional alteration in their metabolism ; and thirdly, there is the possibility, that even when the duodenum is normal and supplying its excitant, and although the pancreas is also normal and yielding, as a result of the action of the excitant, its internal secretion, yet there are changes in the oxidizing tissues such as the liver or muscles which prevent the oxidizing function of these from coming into operation.'

' It is clear that it is only in the first class of case that benefit might be expected to follow in a diabetic from administration of extracts of duodenum, even granting that the experimental difficulties of administration had been so overcome that the active material entered the circulation and reached the pancreas as if it had naturally been formed in the patient's duodenum.'

' Accordingly, it is scarcely to be expected that in all cases administration of extracts of duodenal mucous membrane will cure, or even benefit, diabetics, and to prove the existence of a specific chemical excitant for the internal secretion of the pancreas formed in the duodenum, it is only necessary to show in a fair percentage of cases that abolition of glycosuria follows administration of the extract of duodenal mucous membrane.'

' The three cases recorded in this paper form a commencement in this direction, and, although the number of cases is small, the results are promising, and we publish them in order to attract attention to the subject, and have the matter tested by other observers in a larger number of cases, premising that positive results cannot, for the reasons given above, be expected in all cases.'

Since the publication of the preliminary paper we have tested the effects of duodenal extract in a considerable number of cases, and have had the benefit of reports from other observers employing the treatment.

In the majority of these cases our results have been the same as those recorded by Bainbridge and Beddard in the preceding paper ; that is to say, there has been no appreciable fall in the output of sugar in the urine following the administration of the duodenal extract. In some of these negative cases there has been noticed, however, an improvement in the digestion, and in certain cases the patient's weight has increased.

In a smaller number of cases, we have found a decided drop in the output of sugar after the commencement of the administration of the duodenal extract, which we believe was not to be attributed to change in diet, since the patients were kept upon a constant diet for some considerable time before the treatment was started, and during its continuation.

In some cases there appears to be an escape after a time from the influence of the secretin, as if the pancreas had been temporarily stimulated to greater activity and then had become fatigued or exhausted.

We have not been able, in any case observed since the appearance of our previous paper, to reduce the output of sugar to zero.

With regard to the criticism of Bainbridge and Beddard, that the fall in amount of sugar was due to dieting and not to the action of the duodenal extract, we should like to make the following observations.

The patient in Case I had been in hospital upon a diabetic diet for a period of four months before the administration of the extract was commenced, so that the effect of diet ought by that time to have been completely eliminated, and the dieting was not at all varied until after the sugar had fallen considerably. The patient then left hospital, and the final drop to zero occurred when he was under out-patient treatment and probably not under nearly as strict a diet as while under observation in the hospital.

In Case II, the patient was too ill to defer treatment until the effects of dieting had been established, but the first great fall due to dieting occurred within the first four days, and then a second sudden sharp drop in about three weeks occurred which presented none of the features of a dietetic fall.

Case III was never throughout the treatment put upon a carbohydrate free diet, potatoes (about 3 ounces) being allowed daily, and a small quantity of milk. Also, the patient had been put upon the diet, fully restricted as far as was done throughout, for over a week before the treatment was begun, and the output of sugar had been steady at 3 per cent. for a week before treatment was attempted. Further, the patient reacted rapidly to change in diet which was accomplished in two stages, each accompanied immediately by a fall in the sugar. Although there was during the treatment no change in diet, yet the output of sugar rapidly fell and finally reached zero. Finally, the case proved to be one which was only temporarily stimulated by the secretin, for after the urine had been free of sugar for about a week, it reappeared, although no change had been made in the diet, and was apparently no longer influenced by the secretin, gradually increasing in amount until it reached its former level of about 3 per cent.

The urine in Case No. II of our former paper still remains free from sugar as long as the patient is kept on a carbohydrate-free diet ; over 3 ounces of carbohydrate foods lead to an appearance of sugar, which promptly disappears when the carbohydrate is cut off. No treatment by secretin has been since employed.

In further evidence that the positive effect sometimes obtained with the extract does not stand in relationship to diet, we reproduce a chart showing a fall in amount of sugar in a patient who had been kept in hospital upon a constant diet for several weeks before the commencement of the secretin treatment, and in whom a decrease in output promptly followed administration of freshly prepared acid extract of duodenum, in which the acid was applied as soon as the duodenum could be taken out of the animals (pigs).

The positive evidence in the smaller number of cases appears to us to outweigh the negative evidence in the larger number for the reason above given, that it is only in that class of patients in which the duodenal secretion is at fault that benefit can be expected, and since such cases cannot be diagnosed, it is only by routine employment of the treatment in a considerable number of cases that a final opinion

can be arrived at as to whether cases occur which can be permanently benefited by the treatment.

At present no specific treatment for diabetes is known and only palliative treatment by dieting, and in a lesser degree by drugs, can be attempted.

There exists clear physiological evidence of a connection between the functional activity of the pancreas and diabetes, and also as to the pancreatic activity being influenced by the secretion of the duodenum.

To this there may now be added from the work of Bainbridge and Beddard, that *secretin*, the pancreatic hormone, is absent in many cases of diabetes. Under such conditions, the indication appears to us to be clear that some attempt should be made in cases of diabetes, to supply from without this stimulus usually lacking in the diabetic's duodenum.

If this stimulus could be applied in an efficient fashion at an early stage, before the pancreas began to grow functionless from disuse, and the pancreas were normal otherwise, so that the stimulus did not form a mere whipping up of a feeble gland, then advantage might be expected to follow such stimulation.

In those cases where the pancreas is the seat of organic disease, or is in a condition of atrophy, no such stimulation by the duodenal secretion would, obviously, be of any service.

The observation by Bainbridge and Beddard that in a de-pancreatized dog, which was therefore suffering from severe diabetes, there was still active secretin present in the animal's duodenal mucous membrane, does not seem to touch the question at issue, but rather to illustrate one of those conditions leading to diabetes which would not be affected by secretin treatment. In such an animal, the duodenal stimulant is present, but there is no pancreas to stimulate to the formation of its internal secretion, and the animal becomes diabetic.

The secretin does not directly act upon the diabetic condition, but only by stimulating the pancreas, if the pancreas is absent or diseased, the secretion of the intestine is still formed, but is valueless in the presence of the altered condition of the pancreas.

It is in the reversed condition where the secretin from the patient's own duodenum is lacking or deficient in quantity and the pancreas is normal, that the treatment by secretin becomes desirable.

The observations of Bainbridge and Beddard indicate that in the majority of diabetics, *prosecretin* is absent from the duodenum, and if this is the primary condition in some of these cases, then if secretin could be supplied in similar fashion to that in which it normally enters the circulation at the duodenum, an effect should be obtainable.

We believe that our observations show that in certain cases such an effect has actually been obtained ; that such an effect should always be obtained we have never claimed, in fact in our earlier paper we mentioned that two cases had given us negative results.

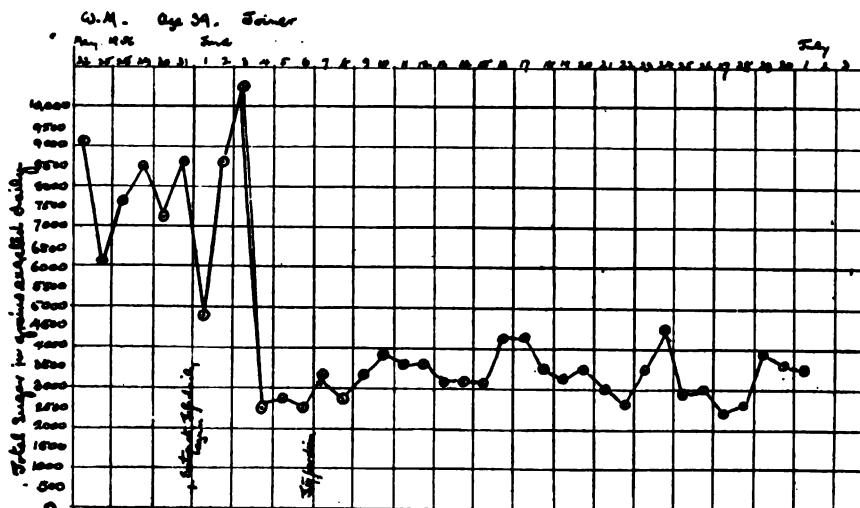


FIG 1.

The chart given above shows the daily output of sugar before and after treatment with an acid extract of duodenal mucous membrane, prepared at once from the duodenum of the pig, taken as soon as possible from the animal after slaughtering and at once placed in acid, otherwise the mode of preparation was identical with that previously described.

The case was one under the care of Dr. Bradshaw in the Royal Infirmary, Liverpool, and we are indebted to him for his kindness in permitting us to publish it, as also to Mr. J. L. Cox, who made the sugar estimations daily by Gerrard's method.

The patient, W. M., a joiner by trade, aged 39, was admitted March 22nd, 1906, complaining of intense thirst, and weakness in the legs, he was much emaciated and stated he had lost two stones in weight during the past month. The urine on admission was 110 ozs. in 24 hours, containing 5.3 per cent. of sugar (23.3 grains per oz.) and acetone and di-acetic acid were present. A sample of blood taken for estimation of the alkalinity of the serum showed lipaemia.

The patient was put on diabetic diet on March 31st and given 1 drachm of sodii bicarb. every four hours, as he was drowsy and had refused food.

No fall whatever in the amount of sugar followed the dieting, but the amount continued to rise slowly.

On April 5th he was put on an extract of duodenum, prepared by the action of acid but the acid was not applied immediately on removal of the duodenum, and the extract was not manufactured in the laboratory. No effect followed this treatment, the sugar continuing to rise and now (April 10th and 11th) standing at 5,500 grains (= 356 grams) daily, instead of at 3,000 grains (= 195 grams) as on admission.

On May 15th the treatment with this acid extract was stopped and the patient was put on codëia,  $\frac{1}{2}$  gr. t.i.d., this drug produced no effect, the sugar continuing to increase and averaging 7,500 grains (= 487 grams) between May 22nd and 30th.

On May 31st he was put on acid extract of duodenum, prepared freshly in the laboratory, the duodenum being placed in acid as soon as possible after removal from the animals (pigs). No change was made in the diet. The movement in total daily amount of sugar is shown on the accompanying chart. On June 31st, the total amount was down to less than 5,000 grains, as against 8,500 on the previous day, the following day it rose to 8,500, and on the next to 10,500, the highest output recorded throughout the case. The following day it dropped suddenly to 2,500, and remained stationary at about that level for three days, and then slowly rose to between 3,000 and 4,000, where it remained until the patient left hospital.

Here we have a patient presenting a severe type of diabetes, who is dieted for nine weeks without effect, and treated with codëia without change in the output of sugar, and upon placing on a properly prepared acid extract of duodenum, there is at once a change in the amount of sugar excreted, the end result being that the total output falls to nearly one-third of the former quantity.

The result may, of course, have been a coincidence, but it is difficult to explain otherwise than in connection with the administration of the extract.

Another case gave a similar effect only less marked, there being here also a preliminary rise followed by a fall from 5,500 grains daily before treatment to 2,800 after treatment.

In another case under the care of one of us and Dr. Calvert of Oswestry, a man, aged 45, under restricted diet, which was not varied during the treatment, gave the results shown in the accompanying chart (Fig. 2). The patient had been under observation for six months

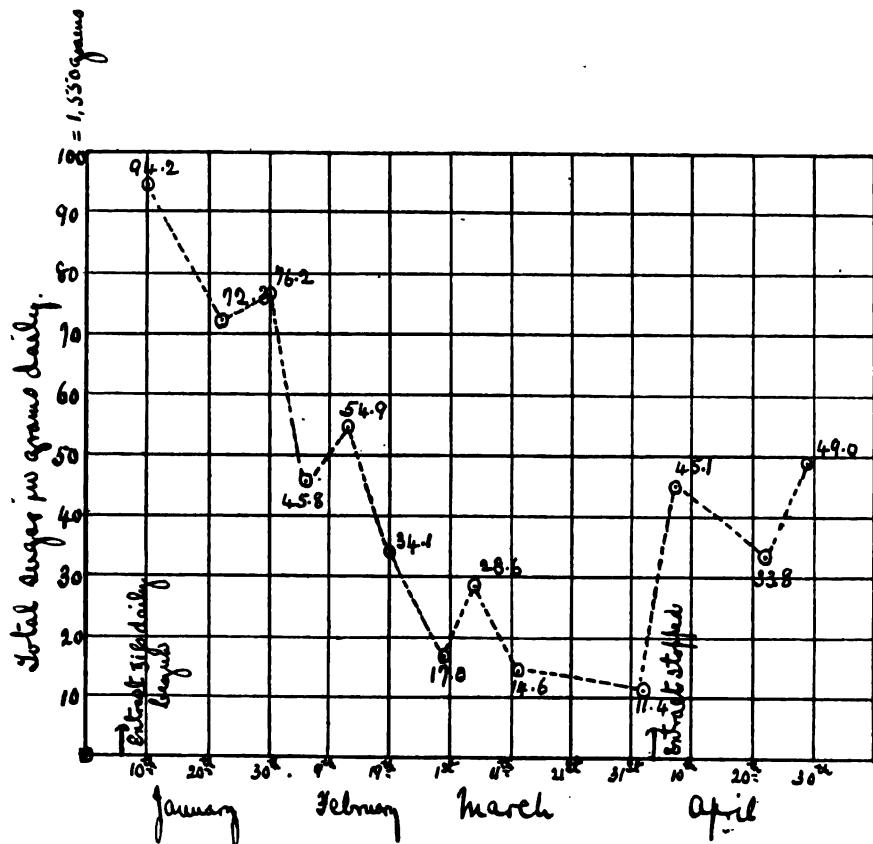


FIG. 2.

before treatment by acid extract was begun and the sugar was not reducible by dieting. Almost at once the dyspepsia from which he was suffering was relieved, and his general nutrition improved to such an extent that he regained over 18 lbs. in weight, which he had previously lost, this improvement was accompanied by a complete recovery of his mental and physical energies.

## ON GLYCOSURIA CAUSED BY EXCESS OF CARBON-DIOXIDE IN THE RESPIRED AIR<sup>1</sup>

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(Received August 8th, 1906)

For the purposes of a research upon the effects on the respiratory exchanges and metabolism of varying the percentage of oxygen in the air breathed, a specially constructed apparatus was devised in which animals under experiment (usually cats) could be kept for as long a period as was desired in an atmosphere containing a constant percentage of oxygen, the carbon-dioxide produced by the animal being absorbed almost as rapidly as it was formed by a large quantity of soda-lime placed in the chamber.

One of the main objects of the enquiry being that of studying, in addition to the gaseous exchanges, the metabolism of the animal as shown by the urine; it was a routine procedure in all cases to examine the urine for abnormal constituents, and in particular for the products of incomplete oxidation, in those cases in which the percentage of oxygen was made less than that in the atmosphere.

Amongst such constituents dextrose was always tested for in the urine, and was invariably found to be absent, even when the animal had been kept in an atmosphere containing 5 to 6 per cent. only of oxygen for a period of several hours, provided that the carbon-dioxide formed during the period had been efficiently removed by the soda-lime placed in the respiratory chamber.

On one occasion, however, the soda-lime failed for some reason to act sufficiently in absorbing the carbon-dioxide, so that at the end of an experiment of 16 hours duration, the respiratory chamber was found to contain 10·3 per cent. of carbon-dioxide and 7·7 per cent.

1. The expenses of this research have been in part defrayed by a grant allotted to Professor B. Moore by the Government Grant Committee of the Royal Society, and the experiments have been carried out under his supervision,

of oxygen. During the period the animal (cat) had passed 130 c.c. of urine, which was found on analysis to contain 3.1 per cent. of sugar. A small amount of urine voided about four hours after the chamber was opened also contained sugar, but after this period the urine ceased to reduce.

This experiment led to the investigations recorded in this paper, which have had for their object the study of whether the glycosuria observed were due to deficiency of oxygen, or excess of carbon-dioxide.

Reference to the literature of the subject showed that the most closely allied observations hitherto recorded were in regard to glycosuria caused by partial asphyxiation. Experiments upon this subject have chiefly been made by observers of the French Schools, Claude Bernard, Dastre, Lépine and Boulud, who came to the conclusion that the glycosuria arose from deficiency of oxygen causing imperfect oxidation, either directly, or by leading to the formation of 'leucomaines' which interfered with the glycolysis (Lépine and Boulud). The effects of an excess of carbon-dioxide, in presence of a normal atmospheric amount of oxygen, or higher, in producing glycosuria was not tested, however, by these observers or their conclusions might have been different.

The fact that in previous experiments of our own in which the percentage of oxygen had been reduced very low (6 per cent. and under) but the carbon-dioxide had been perfectly absorbed, no glycosuria had been obtained, while here, with a somewhat higher percentage of oxygen (7.7 per cent.) in presence of a high percentage of carbon-dioxide (10.3 per cent.) glycosuria in marked degree had resulted, indicated that the glycosuria in our experiment and also that found by previous observers in partial asphyxia, arose from the presence of excess of carbon-dioxide, and not from deficiency of oxygen.

The further experiments given in detail below confirm this view, and show that glycosuria occurs in different types of animal (cat, rabbit and dog) when the percentage of carbon-dioxide in the air breathed rises to 10 to 15 per cent. (the necessary percentage varying somewhat with the animal used), and that this occurs whether the accompanying percentage of oxygen be correspondingly low, or whether the per-

centage of oxygen be maintained at or above the percentage present in the atmosphere.

Before proceeding to a description of the experiments, a brief account may be given of previous experiments upon the effects of partial asphyxiation.

Alvaro Reynoso<sup>1</sup> appears to have been the first to notice glycosuria under such conditions, but made no further accurate observations on the subject.

Claude Bernard<sup>2</sup> found that prolonged asphyxia destroyed the glycogen of the liver, and also that glycosuria was produced.

Dastre<sup>3</sup> who continued Bernard's observations, experimented in a more quantitative fashion, using an apparatus by which the animal could be made to breathe at will either free air, or air from a confined space. He examined the amount of sugar in the blood, and found that this rose from 0·128 per cent. when the animal (dog) was breathing free air, to 0·253 per cent. when breathing was continued for some time in a confined space. The change was ascribed to lack of oxygen in the blood.

Schiff<sup>4</sup> observed that ligature of a limb caused glycosuria and supposed that the coagulation of the blood gave rise to a ferment which increased the production of sugar in the liver.

Lépine and Boulud<sup>5</sup> consider Schiff's explanation improbable, and state that in their opinion it is not the coagulation of the blood but want of oxygen which produces glycosuria by forming 'leucomaines' which interfere with the glycolysis.

These observers state, in support of their view, that injection of the blood of a partially asphyxiated animal into a normal animal (guinea pigs) produces glycosuria in the latter, and insist that this effect is due to 'leucomaines,' produced by lack of oxygen.

It will be observed that the predominant view of the previous observers is that the glycosuria in partial asphyxiation is due to lack

1. Quoted from *Comptes rendus*, Vol. LXXXIX, p. 671.

2. Quoted from *Comptes rendus*, Vol. LXXXIX, p. 669.

3. *Comptes rendus*, Vol. LXXXIX, p. 669.

4. See Lépine and Boulud, *Comptes rendus*, Vol. CXXXIV, p. 582.

5. *Comptes rendus*, Vol. CXXXIV, p. 582.

of oxygen, but throughout there is nothing to show that it might not equally well have arisen from excess of carbon-dioxide in the blood.

This is clearly the case in the earlier experiments where the animal as a whole is partially asphyxiated, for coincidently with the fall in oxygen pressure in the blood there would be a rise in pressure of carbonic acid, and dissolved carbon-dioxide.

It is no less the case in partial asphyxia of a limb produced by ligation, as in Schiff's experiment, for here there would be an accumulation of carbon-dioxide in the blood and tissue fluids of the limb and the same conditions locally as occur over the whole animal in partial asphyxia.

The same may be said about the experiment of Lépine and Boulud of injecting blood from an asphyxiated animal, for this blood would contain both an excess of carbon-dioxide, the product of the previous asphyxiation, and also an excess of sugar set free from the tissues of the asphyxiated animal.

An important point which is shown by the experiments of the present paper is that the amount of sugar secreted in the urine is far in excess of the total amount of sugar capable of circulating in the animal's blood at any one given time, showing that the agent causing the glycosuria is capable of attacking the sugar-containing substances present in the tissues and setting free sugar from these so as to increase the percentage in the blood and lead to glycosuria.

Thus, in Expt. VII, 3, 7·7 grams. of sugar were secreted in about five hours, this, if all present in the animal's blood, would amount to about 3·8 per cent., or more than twenty times the normal amount in the blood, showing that the excess of carbon-dioxide must have led to the setting free of sugar from the sugar-containing substances of the tissues.

The view that the glycosuria is caused by excess of carbon-dioxide and not by lack of oxygen is supported by the work of Hamburger<sup>1</sup> on the effect of passing carbon-dioxide through whipped blood. This observer found that if this were done and then the percentage

1. Osmotischer Druck und Jonenlehre in den medicinischen Wissenschaften, Vol. I, p. 265, 1902.

of sugar in the serum after centrifugalization determined, the percentage was much higher than in the serum of blood which had not been treated with carbon-dioxide before centrifugalizing.

Hamburger ascribes the increased amount of sugar in the serum to the action of the carbon-dioxide in removing sugar from the blood corpuscles, in a similar fashion to that in which it removes alkali and increases the alkalinity of the serum, as shown by Zuntz and confirmed by Hamburger.

Experiments now being carried out in the Bio-Chemical Laboratory, University of Liverpool, by Edie and Spence, which will soon be published in detail, show, however, that there is practically no sugar in the corpuscles, and that the increased percentage of sugar found in serum after the serum or blood has been treated with carbon-dioxide is due to the setting free of sugar, probably from combination with the serum proteins, which would otherwise be lost to the analysis.

These experiments need not be referred to further here, except as an indication of the manner in which carbon-dioxide and probably other causative agents in the production of hyperglycaemia, and hence glycosuria, act in breaking down the formation of compounds in the body between the sugar and constituents of the blood and tissues.

Regarded from this point of view, the glycosuria caused by excess of carbon-dioxide in the air breathed, becomes not a mere additional example of one of the very many causative agents for glycosuria, but gives an indication also of how these many different agents may be regarded as acting in one common way, and producing glycosuria, by acting as detaching agents between glucose and the combining substances which hold it in the blood and tissues.

#### RESPIRATORY APPARATUS USED FOR THE EXPERIMENTS

The apparatus employed was devised to maintain a mixture of gases of approximately constant composition throughout an experiment lasting from several hours to over a day, without a constant circulation of large volumes of air, or mixed air and gas, through the respiratory chamber. Such a circulation, in an experiment lasting

for a long time, demands expensive and complicated apparatus, both for the circulation process itself and for measuring and analysing the gases passing through the chamber. Apparatus of this description lay outside our command, and also it seemed highly desirable for research work in respiration, and accompanying metabolism work, to obtain some simple form of apparatus, which would secure the ends in view. These were approximate constancy in the composition of the atmosphere of the respiratory chamber, including, therefore, some way of keeping percentages of oxygen up, and rapidly removing carbon-dioxide, without sending large volumes of nitrogen through the apparatus ; some means of ready analysis of amount of oxygen used and amount of carbon-dioxide formed ; and a method of collecting urine for metabolism work. The principle employed was to place an absorbent for carbon-dioxide in the chamber, outside the reach of the animal, and as the animal used up the oxygen to have it replaced by oxygen as pure as possible, drawn in automatically as the pressure fell on account of the absorption of carbon-dioxide. As it was found impracticable to obtain oxygen in large quantity above 95 to 96 per cent. pure, it was necessary to arrange a slow measured outflow from the chamber to correct for this, a previous analysis of the percentage of oxygen in the oxygen reservoir gave the rate at which the outflow was to be allowed to take place.

After a good deal of experimentation and modification, the apparatus was arranged as shown in the accompanying sketch in section (Fig. 1) and photograph (Fig. 2).

In the sketch, the respiratory chamber is shown at A. It is a cylindrical vessel made of block tin, with a conical bottom ending in a narrow tube to which a short piece of rubber tube is attached and closed during the experiment by a screw clip. This arrangement serves to collect the urine, which can be drawn off during the experiment by making the pressure in the chamber slightly above atmospheric and keeping the end of the rubber tube below the surface of the urine while it is being drawn off, or it may be collected at the end of the experiment.

The volume of the respiratory chamber was 44 litres.

The animal is placed as shown, upon a perforated circular zinc plate, which serves to retain the faeces ; any particles accidentally dropping through are retained by the smaller meshed little circular plate shown by a dotted line near the draw-off tube.

At F is shown the arrangement for carrying the soda-lime, it is an annular tray of tin made to pass easily down into the chamber and is suspended from the upper rim of the chamber by three slips attached to it at equal distances and turned over at the top so as to hook over the top edge of the chamber. The tray held easily 500 grams, and was charged with about this amount in medium sized granules. The lid was attached in an air-tight fashion by means of

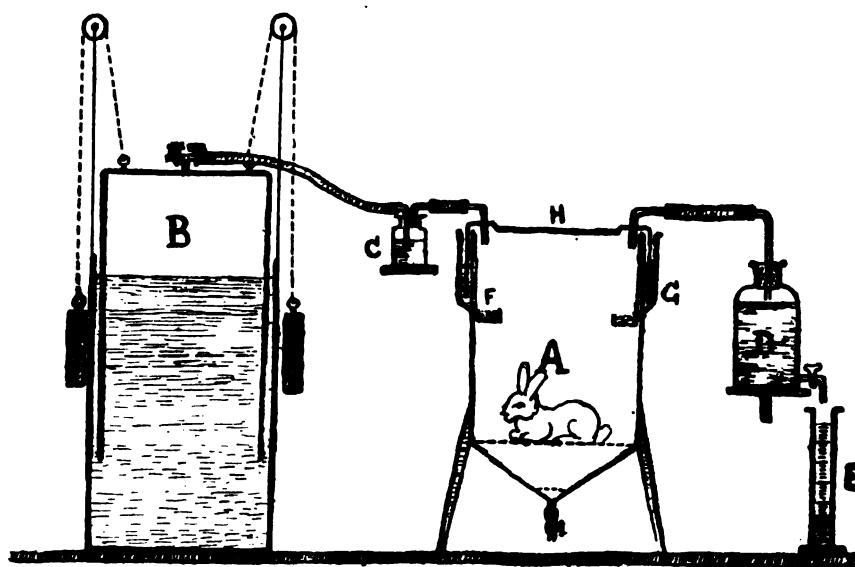


FIG. 1.—Sketch in section of respiratory chamber and accessory apparatus. Respiratory chamber, A; Oxygen reservoir, B; Wash bottle, C; Aspirator, D; Graduated vessel to measure outflow, E; Soda-lime holder, F; Water seal for lid, G; Glass pane for observing animal, H.

a circular water seal, as shown in the sketch. This seal is about 20 centimetres high and is filled half way up with water before the lid is put on. In the centre of the lid is a glass pane, shown at H, by which the animal can be observed during the experiment.

The gasometer B, which had a capacity of 90 litres, was charged

before the experiment with oxygen prepared by heating potassium permanganate.<sup>1</sup> By a side scale, the volume of oxygen contained in it could be determined at the commencement and at any time during the experiment. The suspension weights were so adjusted or weights placed on the top so that the oxygen was just about to bubble through the wash-bottle C. The main use of the wash-bottle C was to control the passage of oxygen, and to act as an index to show the rate at which the animal was using oxygen, and that the experiment was proceeding satisfactorily.

The arrangement shown by D and E was necessitated by the fact that it was not found practicable to prepare oxygen in the quantities required in a quite pure state. Oxygen prepared with moderate care from potassium permanganate, taking care to flush all the air out before starting to collect, contains 95 to 96 per cent. of oxygen, the remainder being nitrogen. If, therefore, the animal were allowed to draw over this mixture as it used up the oxygen contained in the chamber, and the carbon-dioxide was absorbed by the soda-lime, then on account of the small percentage of nitrogen drawn in, the oxygen content of the chamber would slowly fall and the nitrogen content rise as the experiment progressed. In order to correct this the aspirator, D, is turned on so as to run very slowly, and the amount run off in a given time is measured in the graduated vessel, E. It is easy to calculate the rate at which gas must be drawn into D in order to keep the percentage of oxygen in A constant, knowing the percentage of oxygen in A and in B. Suppose that B contains 95 per cent. of oxygen and 5 per cent. of nitrogen, then, if all the gas going into D were supposed to be nitrogen, it is evident that for every 1,000 c.c. leaving B, 50 c.c. of nitrogen would enter A and have to be drawn over into D to keep a constant level; but A contains oxygen as well as nitrogen and this must be allowed for. Suppose in any experiment A is to contain 20 per cent. of oxygen, then in 100 c.c. of A there are 80 c.c. of nitrogen, therefore to get 50 c.c. of nitrogen over into D, there must be run off

1. The oxygen sold in compressed condition in cylinders only contains 88 to 90 per cent. of oxygen, and is much too poor in quality for the purpose.

$50 \times \frac{100}{80}$  c.c. = 62.5 c.c. That is to say, in order to keep the percentage of oxygen in A constant under the above conditions, for every 1000 c.c. taken in from B, 62.5 c.c. must be run off into D. In general terms, if  $V$  be the volume coming from A,  $v$ , the volume run off into D,  $x$ , the percentage of oxygen in B, and  $a$ , the percentage of oxygen required to be maintained in A, then  $v = V \times \frac{100 - x}{100 - a}$ . It follows that with a capacity of 80 litres in B, for the maintenance of 20 per cent. of oxygen in A, a capacity of about 5 litres is required in D. For a lower required percentage in A, a slower flow into D, and for a higher percentage a more rapid flow are required.

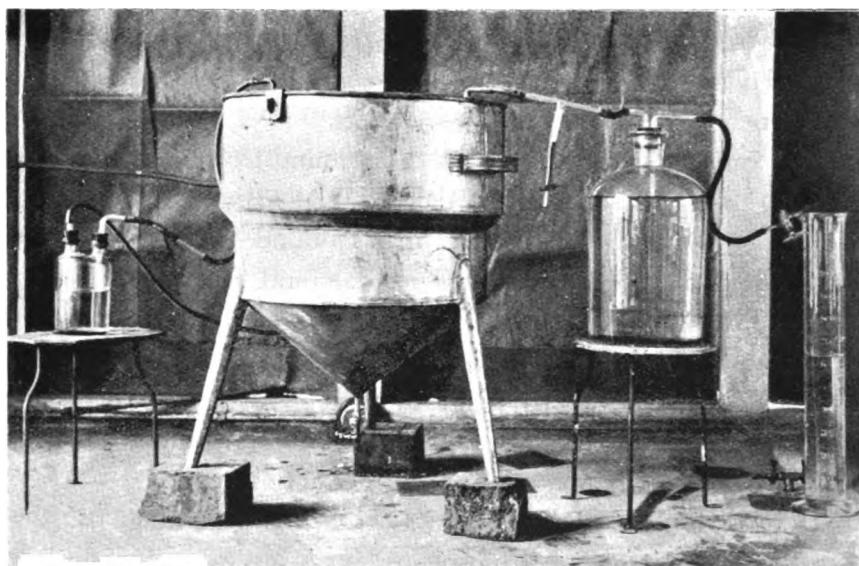


FIG. 2.—Photograph showing respiratory chamber and aspirating apparatus for adjusting percentage of oxygen in it. The lead pipe seen passing off at the left-hand border goes to the oxygen reservoir.

Two slight modifications, not shown in the sketch were employed in actual use of the apparatus, these consisted in the insertion of a T piece on each of the short rubber joints between C and A, and D and A respectively. The first of these, between C and A, served for the insertion of a water manometer to show the pressure in A, the second, between D and A, served to draw off samples of the gas in A for analysis during the experiment. The free end of the latter

T piece was normally kept closed by a screw clip, which was opened after the gas burette had been attached, and after filling and re-emptying the burette several times so as to obtain a fair sample, the clip was re-applied and the burette withdrawn. A few minutes is enough for determining in the sample of gas so taken the percentages of carbon-dioxide and oxygen, and if the latter is different from what it ought to be, a modification is made in the rate of drawing off through D, in order to compensate.

Soda-lime was found to be the only efficient absorbent for carbon-dioxide in those experiments where it was desired to keep this low; with soda-lime acting well, it is possible to keep the carbon-dioxide down to 0.2 per cent. and lower. Strong caustic potash solution was tried with as large a surface as possible but was found entirely inadequate.

The amount of oxygen used is easily found from the volume of B at the beginning and end, and initial and final analysis of the air in the system A + D, and a knowledge of its volume. To determine the amount of carbon-dioxide formed, the amount in A + D is found at the end of the experiment by a gas analysis, and this is added to the amount absorbed by the soda-lime, which is found by analysis of a weighed portion after thoroughly mixing to obtain an average specimen. A control analysis of the soda-lime, previous to use, must also be carried out, and the necessary deduction made.

If it is desired to work far away from the percentage of oxygen normally present in the atmosphere, it is necessary to flush the respiratory chamber out rapidly at the commencement of the experiment. If a higher percentage of oxygen than atmospheric is being used, the chamber can be flushed out with oxygen from an accessory reservoir, or part of the oxygen in B may be used for the purpose. In carrying out this operation, extra weights are placed on the reservoir and the screw clip at the bottom of the respiratory chamber is opened, while the rubber tube is kept under water in a beaker, and a rapid stream of gas is bubbled off until a sample of air taken from A shows the desired percentage composition. After this the experiment is run as above described.

For running the mixture in A down to a low percentage in oxygen, an accessory reservoir full of nitrogen is used, connected up in the same way as B is shown in the sketch, and a rapid stream sent through as above described in the case of oxygen, until a sample shows the composition desired.

After considerable experimentation it was found that the best way to prepare nitrogen in large amounts was by passing air containing ammonia vapour over palladium asbestos in a combustion tube, heated by a Bunsen burner. The air is bubbled by means of a water pump through a bottle containing *strong* ammonia, and the nitrogen washed from nitric acid or excess of ammonia by passing through wash bottles containing (a) water or dilute acid, and (b) caustic soda. The palladium asbestos acts only catalytically and is much better than copper foil which requires a good deal of adjusting, a plug of a few centimetres loosely packed is enough. When the process starts, it generates almost enough heat to go by itself, the palladium asbestos glowing a bright red. If there is not enough ammonia a good deal of nitric acid is formed, and we have found that the ammonia is almost quantitatively converted into nitric acid ; but this can be obviated by using strong ammonia in the bottle through which the air is bubbled before passing over the palladium asbestos. Under such conditions all the oxygen is burnt out and one obtains both the atmospheric nitrogen and the nitrogen of the ammonia, and 80 to 100 litres can easily be made in three to four hours with a single combustion tube in use.

The respiratory apparatus is fairly efficient for the class of experiment for which it was intended, of studying metabolism and gaseous exchange when different percentages of oxygen are present in the air breathed and the percentage of carbon-dioxide is kept at a low level, and it further presents the advantage of giving the materials to be analysed in a comparatively small volume.

It is intended in the future to use it for this purpose, and to determine the lowest percentage of oxygen at which life is possible for long periods, and the effect of such low oxygen pressures upon metabolism.

But for the purpose to which the discovery of the production of glycosuria by excess of carbon-dioxide in the air breathed led of studying the effects of different percentages of carbon-dioxide, it is not possible to use the apparatus without dispensing with the special advantage of not having to ventilate with considerable volumes of gaseous mixtures.

It was necessary, therefore, to abandon the exact study of the gaseous exchanges in presence of different percentages of carbon-dioxide, as we did not possess exact gas meters, and to study only the effects of such percentages in producing glycosuria, and the level of carbon-dioxide pressure at which such glycosuria appeared.

At first an attempt was made to limit the percentage of carbon-dioxide by means of using a quantity of soda-lime presenting an inadequate surface to give complete absorption, but it was found impossible to preserve even an approximate percentage of carbon-dioxide in this way, as the soda-lime absorbed too rapidly at first and then fell off as the experiment progressed, so that the pressure of carbon-dioxide continually increased.

The plan was then followed of leaving out the soda-lime entirely and adding, at the commencement of the experiment, carbon-dioxide, generated in a Kipp by action of acid on marble, until an analysis showed that the desired percentage of carbon-dioxide had been obtained, oxygen also being fed in, initially, where it was desired to keep the percentage of oxygen at, or above, the atmospheric amount. In this way, it was possible to soon get within the respiratory chamber a mixture containing a high percentage of carbon-dioxide (10 or 15 per cent. or higher), and at the same time as high a percentage of oxygen as in atmospheric air, or higher if so desired. This state of affairs having been established, the next procedure is to maintain it so with as little variation as possible throughout the experiment. For this purpose the bottle D was removed, and the apparatus joined here to a Bunsen's water pump, while the water manometer, previously mentioned as occupying the free end of the T piece between C and A, was also removed, and a bottle similar to C, communicating with the atmospheric air in a similar way to that in which C itself

communicated with the chamber B, was inserted in its stead. Two screw-down clips were then placed, one between B and C, and the other between the new wash-bottle and the atmosphere. In this way the total amount of mixed air and oxygen fed into A could be regulated by the speed at which the water pump was worked, and accordingly the amount of carbon-dioxide removed from the chamber could be altered and made equal to the rate at which the animal produced carbon-dioxide; further, by varying by means of the two screw clips, the relative rate at which air was let in through the new wash-bottle or oxygen through the wash-bottle C, the percentage in oxygen of the mixture admitted could be varied and so that in the chamber A, so that this gave an adjustment for preserving the oxygen percentage constant at any given level. Frequent samplings of the air of the chamber are necessary and corresponding adjustments of inlet and outlet, but with care approximately constant percentages of oxygen and carbon-dioxide can be maintained.

These adaptations could be much simplified by the placing in the circuit of three good meters, one on the outlet and one each upon the air inlet and oxygen inlet, but unfortunately, these were not at our command.

#### DESCRIPTION OF EXPERIMENTS

The experiments may be divided into those in which the percentage of oxygen was low and the percentage of carbon-dioxide high; those in which the percentage of oxygen was low and the percentage of carbon-dioxide kept at a minimal value or practically absent; and those in which while the pressure of oxygen was kept as high as in the atmosphere or higher, the percentage of carbon-dioxide was also high.

The evidence obtained by so varying the percentages of the two gases, shows that glycosuria occurs after the carbon-dioxide passes a certain percentage, amounting to 10 to 15 per cent., whether the oxygen pressure is high or low, and that low oxygen percentage, apart from accumulation of carbon-dioxide, does not induce glycosuria.

*Experiment 1.—Low percentage of oxygen and high percentage of carbon-dioxide.* A cat of 2·6 kilograms was placed in the respiratory chamber with an insufficient amount of

soda-lime to absorb all the carbon-dioxide. The experiment lasted for sixteen hours, and the gases of the chamber at the end of the experiment showed 77 per cent. of oxygen and 10.3 per cent. of carbon dioxide. During the period the animal was in the chamber, 130 c.c. of urine was passed containing 3.1 per cent. of glucose.

*Experiment II.—Low percentage of oxygen, but carbon-dioxide kept at as low a value as possible.* A cat of 2.2 kilograms was used, the urine being previously tested and found free from sugar. Soda-lime in sufficient amount to absorb all the carbon-dioxide produced was put in, but instead of allowing oxygen to be sucked in to the partial vacuum caused by the absorption, a supply of nitrogen was allowed to pass in until the percentage of oxygen had fallen to about 7 per cent. A mixture of air and nitrogen was now aspirated through the chamber so as to keep the oxygen in the air breathed at between 5 and 6 per cent. for about four hours.

Analyses throughout the experiment gave the following results:—

Commencement at 2.10 p.m.	0.22 per cent. CO <sub>2</sub> ,	6.9 per cent. O <sub>2</sub>
„ 3.25 „	0.33 „ „ „	5.9 „ „ „
„ 4.30 „	0.45 „ „ „	5.0 „ „ „
„ 5.30 „	0.56 „ „ „	5.7 „ „ „
„ 6.30 „	0.44 „ „ „	5.3 „ „ „

Here, the animal was subjected to a lower percentage of oxygen than in the previous experiment for over four hours, yet, in the absence of the high percentage of carbon-dioxide, the animal suffered no inconvenience and no glycosuria whatever resulted, 50 c.c. of urine being obtained free from sugar.

*Experiment III.—High percentage of oxygen accompanied by high percentage of carbon-dioxide.* A cat of 2.9 kilograms, the urine of which was free from sugar, was placed in the chamber and the percentage of carbon-dioxide was raised as rapidly as possible to 18 per cent., while at the same time, the percentage of oxygen was kept up by aspirating a mixture of carbon-dioxide and oxygen through the chamber. A mixture of air and oxygen with a little carbon-dioxide was now aspirated for a period of 5½ hours. During this period the percentage of oxygen never fell below 21 per cent., that of the carbon-dioxide varied between 18 and 22.5 per cent., as shown by the following table:—

Commencement at 11.45 a.m.	20.0 per cent. CO <sub>2</sub> .	21.3 per cent. O <sub>2</sub>
„ 12.45 p.m.	22.7 „ „ „	21.1 „ „ „
„ 2.0 „	18.1 „ „ „	25.1 „ „ „
„ 3.0 „	20.0 „ „ „	22.1 „ „ „
„ 4.15 „	18.2 „ „ „	21.2 „ „ „

Experiment finished at 5 p.m.

No urine was passed during the experiment, but next morning, at 9 a.m., 225 c.c. of urine were obtained containing 1.1 per cent. of glucose. Total amount of sugar 2.48 grams, which is far in excess of the quantity which could be normally present in the animal's blood. The next sample of urine obtained was free from sugar.

Here, the percentage of oxygen throughout the experiment was at or above the amount present in atmospheric air, and yet the high percentage of carbon-dioxide led to glycosuria.

It may be noted that the animal was anaesthetised by the carbon-dioxide throughout the experiment.

*Experiment IV.—High percentage of oxygen, with high percentage of carbon-dioxide in the rabbit.* In this experiment a rabbit of 2·18 kilograms was placed in the chamber with no soda-lime. A mixture of carbon-dioxide and oxygen was aspirated through the chamber so as to raise the percentage of carbon-dioxide rapidly to 17 per cent., at the same time keeping the oxygen at least up to the atmospheric percentage. Air and oxygen were then aspirated through. The experiment was kept up for 5½ hours, the animal breathing during this time 18·7 to 27·4 per cent. of carbon-dioxide, and 21·1 to 25 per cent. of oxygen as shown by the following table of analyses :—

Commencement at 12 noon	11·2 per cent. CO <sub>2</sub> , 23·7 per cent. O <sub>2</sub>
„ 1 p.m.,	18·7 „ „ „ 25·1 „ „ „
„ 2·10 „	27·4 „ „ „ 22·6 „ „ „
„ 3·30 „	24·1 „ „ „ 21·1 „ „ „
„ 4·45 „	21·5 „ „ „ 23·4 „ „ „
„ 5·30 „	23·1 „ „ „ 21·9 „ „ „

At the termination of the experiment 32 c.c. of urine were obtained, containing 2·0 per cent. of sugar. No urine had been passed next morning nor was passed during that day, but at the end of 36 hours, 107 c.c. were found, which was free from sugar.

Although the percentage of carbon-dioxide was as high in this case as in the preceding experiment with the cat, the rabbit was not anaesthetised by the carbon-dioxide ; it sat up during the experiment and did not appear to be in any way inconvenienced by the high percentage of carbon-dioxide.

*Experiment V.—High percentage of oxygen, with high percentage of carbon-dioxide in the dog.* A dog of 3·2 kilograms was placed in the respiratory chamber and the experiment was carried out exactly as above described in the case of the rabbit. The percentage of oxygen was practically that in the atmosphere during the experiment, which lasted for about three hours, while carbon-dioxide varied from 17·7 to 20·8 per cent.

The animal was completely anaesthetized and took about three-quarters of an hour to come completely round after removal.

Commencement at 11.25 a.m.	19·2 per cent. CO <sub>2</sub> , 22·3 per cent. O <sub>2</sub>
„ 12·15 p.m.	20·8 „ „ „ 19·0 „ „ „
„ 1·20 „	17·7 „ „ „ 22·5 „ „ „
„ 2·15 „	20·0 „ „ „ 20·1 „ „ „

No urine was present at the close of the experiment at 2·30 p.m., but at 4·30 p.m. 85 c.c. were passed which contained 1·81 per cent. of sugar. The next quantity of urine passed contained no sugar.

*Experiment VI.—High percentage of oxygen, but less than adequate amount of carbon-dioxide to produce glycosuria.* This experiment and the succeeding one are given to show the limit of pressure of carbon-dioxide at which glycosuria begins to be produced, which lies between 10 and 15 per cent. The percentage varies somewhat with the animal; thus in Expt. I it was obtained with slightly over 10 per cent., in another animal 10 to 12 per cent. failed to give glycosuria, but 15 per cent. or over never failed to cause glycosuria.

The animal, a cat, weighing 2·6 kilograms, was placed in the chamber with no soda-lime present, at 2.15 p.m., and a current of air and oxygen, mixed, was aspirated slowly through the chamber.

Commencement at 2.15 p.m.	0·00 per cent. CO <sub>2</sub> , 21 per cent. O <sub>2</sub>
„ 5.15 „	5·1 „ „ „ 22 „ „ „
„ 6.15 „	6·9 „ „ „ 20·2 „ „ „
„ 8.30 „	8·2 „ „ „ 15·1 „ „ „
„ 9.20 „	7·9 „ „ „ 17·2 „ „ „

No sugar was found in the urine as a result of the experiment.

In another experiment with this same animal, in which the carbon-dioxide reached 12·4 per cent., 120 c.c. of urine were obtained with slightly over 1 per cent. of sugar.

*Experiment VII.—Amount of carbon-dioxide kept below ten per cent., but oxygen percentage allowed to fall.* The animal, a cat, weighing 2·9 kilograms, was placed in the chamber, soda-lime absent, and the CO<sub>2</sub> formed in its own respiration allowed to increase to about 8 per cent., when a current of air was aspirated through.

At 12.25 p.m.	7·9 per cent. CO <sub>2</sub> , 9·4 per cent. O <sub>2</sub>
1.35 „	9·3 „ „ „ 7·2 „ „ „
2.45 „	6·8 „ „ „ 11·2 „ „ „
4.45 „	8·4 „ „ „ 9·3 „ „ „
6.00 „	9·9 „ „ „ 7·2 „ „ „

No sugar was found in the urine.

In a subsequent experiment with the same animal, in which the percentage of carbon-dioxide varied between 13·3 and 14·5 per cent., a good reduction was obtained and sugar was shown to be present by the fermentation test.

In a third experiment with the same animal, in which the carbon-dioxide throughout the greater part of the experiment was kept at the high level of 17 to 21·9 per cent., and the oxygen, although less than atmospheric value, was still very much higher than in the above negative experiment, the highest percentage of sugar in the series was obtained, viz., 5·42 per cent. in 142 c.c., followed by 97 c.c. with 1·03 per cent., giving a total amount of 8·7 grams. The animal was anaesthetized throughout by the carbon-dioxide.

For contrast with the above experiment the following details are given.

A mixture of carbon-dioxide and oxygen was first passed in and then followed by a mixture of air and oxygen.

At 11.40 a.m.	14·6 per cent. of CO <sub>2</sub> ,	20 per cent. of O <sub>2</sub>
12.5 p.m.	17·0	17·6
1.5 "	19·5	17·4
2.0 "	19·8	13·8
3.20 "	19·5	12·5
4.35 "	21·9	10·2
5.15 "	21·4	9·4

The percentage of carbon-dioxide required to produce the glycosuria in these experiments may appear to be a high one, viz., 10 to 15 per cent., but it must be remembered that this is a percentage by volume in a gaseous mixture, and that the corresponding concentration of the carbon-dioxide regarded as dissolved to form a solution would not be very high. Thus a normal solution being the molecular weight dissolved in a litre would have a pressure of 22·3 atmospheres, and hence a partial pressure of 15 per cent. of an atmosphere, which invariably produces glycosuria, would amount to  $\frac{1}{22·3} \times \frac{15}{100}$  normal =  $\frac{1}{148}$  N.

The production of glycosuria by the presence in the blood of such a small amount of weak acid like carbonic acid, suggested the idea that the sugar in diabetes might possibly be detached from combination with the proteid of the cells, by the agency of the weak organic acids such as  $\beta$ -oxy-butyric, and diacetic acid, which are commonly present in the blood in that disease.

It is exceedingly difficult to test this hypothesis experimentally, because it is necessary to anaesthetize in order to collect the urine, and to get the acid injected intravenously, and anaesthetization with chloroform, ether, or other anaesthetic, gives rise to a copious glycosuria, in presence of which it becomes almost impossible to judge what effect is being produced by the acid injection. As far as it was possible to judge the effect was negative, no increase of glycosuria resulting, but in some cases an apparent diminution. The question may, however, be one of dosage, and a *small* percentage of dilute organic acid, in the absence of other causative agent for glycosuria, might have a different result to a larger dose when glycosuria is already present. In connection with the anaesthetizing action of

the carbonic acid in the case of the cat and dog, it is interesting to note that all anaesthetics which have been tested to that end have been shown to produce glycosuria, such as ether, chloroform, nitrous oxide, morphia, etc.—there is no known exception to the rule.

If it be remembered that all the anaesthetics, as shown by Moore and Roaf,<sup>1</sup> possess the property of adsorbing and combining with the tissue proteins and in this way limiting the activity of bioplasm and its power of combining with bodies for the purposes of metabolism, as also with inorganic constituents, an easy explanation becomes apparent for the general effect of all anaesthetics in producing glycosuria.

There is little doubt that the carbohydrate of the cell is retained by a process of combination or adsorption with the protein of the cell. If, now, the protein is simultaneously offered any anaesthetic for combination and has a stronger attraction for this, in all probability the carbohydrate previously held in combination will be set free, and the percentage of sugar in the blood will be raised and lead to glycosuria.

The action of carbon-dioxide, ether and chloroform upon the carbohydrate of the serum, blood, and tissues, as an agent in producing glycosuria is at present being investigated by Moore, Edie, and Spence, and will be described in a separate paper.

#### SUMMARY

1. The glycosuria found after partial asphyxiation, is not due as hitherto supposed to lack of oxygen, but to the high percentage of carbon-dioxide in the respired air.
2. The presence of ten to fifteen per cent., by volume, of carbon-dioxide in the respired air leads to glycosuria, and this occurs even if the percentage of oxygen be over that present in the atmospheric air.
3. A low percentage of oxygen alone (less than six per cent.) unaccompanied by excess of carbon dioxide never produces glycosuria.
4. The high percentage of carbon-dioxide necessary to produce glycosuria causes also in the cat and dog, complete anaesthesia ; in the

1. *Proc. Roy. Soc.*, Vol. LXXIII, p. 382, 1904 ; and B, Vol. LXXVII, p. 86, 1905. See also Edie, *Thompson Yates and Johnston Labs. Reports*, Part i, p. 195, 1905.

rabbit, glycosuria appears before anaesthesia, whether a higher percentage of carbon-dioxide would also produce anaesthesia in this animal was not observed.

5. Carbon-dioxide, accordingly, falls into the general rule that all anaesthetics produce glycosuria.

6. Since it is known that anaesthetics combine with proteins, this suggests that the glycosuria is due to combination occurring between anaesthetic and protein, and causing the setting free of carbohydrate from previous combination with protein.

## TRYPSIN AND ANTITRYPSIN

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In a previous paper<sup>1</sup> I have given an account of experiments on the antitryptic action of serum-albumin, the main results of which were :—

(1) If the trypsin and the antibody are mixed before they are added to the substrate, then the neutralising effect of the antibody is larger than if they are added separately. (2) The effect increases up to a certain point, when the trypsin and the antibody are kept in contact before the addition of the substrate. (3) The maximum effect of the antibody is larger the higher the temperature. The effect corresponding to a certain temperature increases if the temperature is raised, but remains the same if the temperature is lowered.

The continuation of these experiments is given in the following paper. The estimation of the digesting power was carried out by precipitating the samples to be compared with equal volumes of tannic acid solution and determining the nitrogen in equal volumes of the filtrates<sup>2</sup>. Therefore the figures given represent the number of cubic centimetres of decinormal acid, corresponding to the nitrogen in equal volumes of the tannic acid filtrate. In all experiments the antibody was allowed to act upon the trypsin before the substrate (casein) was added. It may be pointed out that the proteid in the antibody solution (the serumalbumin dialysed free from salt) is practically indigestible, unless a very large excess of trypsin is used. An amount of trypsin, which with an excess of casein would have given the effect 90, gave with 10 c.c. of antibody the effect 0'2.

1. *Journ. of Physiol.*, Vol. XXXII, p. 390, 1905.

2. The tannic acid solution contained in two litres: 200 grs. tannic acid, 100 c.c. glacial acetic acid, 50 grs. sodium chloride, and 50 grs. sodium acetate.

THE INFLUENCE OF THE CONCENTRATION OF THE TRYPSIN-  
ANTITRYPSIN MIXTURE ON THE AMOUNT OF  
TRYPSIN NEUTRALISED.

That concentration effects no material influence is borne out by the following experiments :—

*Experiment I.*—In this experiment the ratio between the concentrations to be compared was 2 : 1. Two sets of specimens containing the one (A) 50 c.c. tryps. + 10 c.c. (antib. +  $H_2O$ ), and the other (B) 50 c.c. tryps. + 10 c.c. (antib. +  $H_2O$ ) + 60 c.c.  $H_2O$ , were prepared and kept at 21°. After the intervals of time recorded 10 c.c. of the specimens of set A, together with 10 c.c. of water, were added to 50 c.c. of casein solution. Of the specimens of set B, 20 c.c. were added to the same amount of casein. After 24 hours at 37°, the nitrogen was determined in equal volumes of the tannic acid filtrates.

		20 min.	4 hours.	24 hours.
0.625 c.c. antib.	A	36.05	36.25	—
	B	36.35	35.9	—
1.25	A	32.65	33.2	—
	B	32.45	32.65	—
2.5 c.c.	A	20.0	20.1	—
	B	19.55	19.2	—
5 c.c.	A	8.2	6.25	6.0
	B	8.4	6.75	5.5
10 c.c.	A	5.35	4.55	4.4
	B	5.4	4.8	4.15

In the specimens with 0.625, 1.25 and 2.5 c.c. of antibody, the final state of equilibrium was reached in 20 min., but not in those with larger amounts of antibody. In no case can any influence of the concentration be observed.

*Experiment II.*—Ratio between concentrations 4 : 1. Mixtures kept at 37°.

- (A) 20 tr. + 100 antib.
- (B) 20 tr. + 100 antib. + 360  $H_2O$ .

Of A, 25 c.c., and of B, 100 c.c. were taken for digestion as above.

	5 min.	30 min.	1 Hour
A	4.6	3.8	3.75
B	4.85	4.4	4.2

Of the same agents another two mixtures were prepared :

(A) 20 tr. + 1 antib.  
(B) 20 tr. + 1 antib. + 63 H<sub>2</sub>O.

and 5 resp. 20 c.c. taken for digestion.

	5 min.	30 min.
A	32.95	32.45
B	32.4	32.05

It must be borne in mind that Experiments 1 and 2 were carried out with such small amounts of casein that the results are not proportional to the amounts of active trypsin present,<sup>1</sup> and therefore, the results do not allow of any conclusions as to the relative amounts of trypsin neutralised by different amounts of antibody.

#### CAN ANY OF THE AGENTS (TRYPSIN OR ANTIBODY) BE COMPLETELY NEUTRALISED ?

My investigations on this subject show, that when little antibody is used, then the antibody can be completely neutralised, as proved by the fact that fresh trypsin added develops its full activity. In the cases tried more than three-quarters of the whole amount of trypsin could be neutralised without leaving any free antibody. On the other hand, if much antibody is used, then some antibody remains free, the fluid showing strongly antitryptic action upon added trypsin ; at the same time the fluid manifests some tryptic activity even after 24 hours in presence of very large amounts of antibody. In short, *the antibody can be completely neutralised by using a sufficient amount of trypsin, whilst it is impossible to neutralise all trypsin by an excess of antibody.*

Two series of experiments bearing on this subject are recorded below. In these, such an excess of casein was used that the tryptic effect was proportional to the active trypsin present.<sup>2</sup> This was proved to be the case by the following experiment, where the effects of 2.5 and 5 c.c. of trypsin were found to be 25.7 resp. 51.7. Therefore all figures less than 51.7 are inside the range of proportionality.

1. Hedin, 'Observations on the action of trypsin,' *Journ. of Physiol.*, Vol. XXXII, p. 468, 1905.

2. *Journ. of Physiol.*, Vol. XXXII, pp. 471-474, 1905.

*Experiment III.*—Each specimen contained 300 c.c. casein + 10 c.c. (tryps. + H<sub>2</sub>O) + 1 c.c. (antib. + H<sub>2</sub>O). The trypsin or, in case the trypsin was added in two lots, the first lot of it, the antibody and the water were kept for two hours at 37°, then the second lot of trypsin was added, and all specimens were kept for another hour at 37° before the casein was added. The digestion lasted for 24 hours. 150 c.c. tannin acid solution and 300 c.c. filtrate were taken for each analysis.

1.	2·5 c.c. tryps. without antibody	...	...	...	...	25·7
2.	5 c.c. "	...	...	...	...	51·7
3.	5 c.c. " + 0·125 c.c. antib.	...	...	...	...	21·55
4.	5 c.c. " + 2·5 c.c. tryps	...	...	...	...	47·35
5.	5 c.c. " + 0·25 c.c. antib.	...	...	...	...	11·95
6.	5 c.c. " + 2·5 c.c. tryps.	...	...	...	...	36·9

The effect of an addition of 2·5 c.c. of trypsin is 47·35 — 21·55 = 25·8 in Specimen 4, and 36·9 — 11·95 = 24·95 in Specimen 6, as against 25·7 in Specimen 1 without antibody, showing that in both cases the antibody was completely engaged before the second lot of trypsin was added.

*Experiment IV* was carried out like Experiment III, only with another enzyme.

1.	5 tryps. without antibody	...	...	...	...	...	35·85
2.	" + 0·25 antib.	...	...	...	...	...	8
3.	" + 5 tryps.	...	...	...	...	...	43·25
4.	" + 0·5 antib.	...	...	...	...	...	6·6
5.	" + 5 tryps.	...	...	...	...	...	17·75
6.	" + 0·75 antib.	...	...	...	...	...	6·65
7.	" + 5 tryps.	...	...	...	...	...	8·95
8.	" + 1 antib.	...	...	...	...	...	6·55
9.	" + 5 tryps.	...	...	...	...	...	8·75

Specimen 3 shows that the addition of 5 c.c. of trypsin to Specimen 2, gives the effect 43·25 — 8 = 35·25, or practically the same effect as 5 tryps. without the presence of antibody (35·85 in Specimen 1). On the other hand, Specimens 4-9, which all contained more antibody than 0·25 c.c., show that the effect of the second lot of trypsin was not nearly the same as without antibody. Therefore, in Specimens 5, 7, 9, there was active antibody present, when the second lot of trypsin was added.

*Experiment V* was carried out with large amounts of antibody and long time of interaction of the agents. The specimens contained :

100 c.c. cas. + 12 c.c. (tryps. + H<sub>2</sub>O) + 3 c.c. (antib. + H<sub>2</sub>O).

Trypsin and antibody were kept at 37° for 24 hours, then the second lot of trypsin and the casein were added. After 24 hours' digestion, 50 c.c. tannic acid solution were added and 100 c.c. filtrate were taken for analysis.

1.	2 tryps. without antibody	...	...	...	...	...	...	9.0
	Therefore 10 c.c. tryps. corresponds to	...	...	...	...	...	...	45.0
2.	10 tryps. + 1 antibody	...	...	...	...	...	...	2.1
3.	" " + 2 tryps.	...	...	...	...	...	...	4.9
4.	" " + 2 antib....	...	...	...	...	...	...	1.95
5.	" " + 2 tryps.	...	...	...	...	...	...	2.55
6.	" " + 3 antib.	...	...	...	...	...	...	1.95
7.	" " + 2 tryps.	...	...	...	...	...	...	2.45

The figures obtained indicate that in Specimens 2, 4, 6 there was free trypsin and free antibody present at the same time.

#### THE AMOUNT OF TRYPSIN NEUTRALISED BY A CERTAIN AMOUNT OF ANTIBODY

Experiments bearing on this point show, that when different amounts of antibody are made to act upon the same amount of trypsin, then *the effect of a small amount is always greater per unit of antibody*. In the experiments such an amount of casein was used that the results obtained were proportional to the active amount of trypsin present.

*Experiment VI.*—This experiment is the same as Experiment III, only with the results differently arranged and with an additional set of specimens containing 10 c.c. of trypsin.

1.	5 tryps. + 0 antib.	...	51.7	4.	10 tryps. + 0 antib.	...	103.4 <sup>1</sup>
2.	" + 0.125 "	...	21.55	5.	" + 0.25 "	...	44.1
3.	" + 0.25 "	...	11.95	6.	" + 0.5 "	...	25.95

According to Experiment III, the Specimens 2 and 3 contained no free antibody. The same, therefore, must have been the case with 5 and 6, which had the double amounts of agents and gave approximately the double effect. Therefore, on complete binding, the

1. Since this figure lies outside the range of proportionality, it has been calculated by doubling the effect of 5 c.c. of trypsin.

neutralising effect of 0.125 c.c. of antibody is found to be 30.15 from 1 and 2, and 9.6 from 2 and 3, and for 0.25 c.c. of antibody one obtains the effect 59.3 from 4 and 5, and 18.15 from 5 and 6.

*Experiment VII* is the same as Experiment IV, with an additional set of specimens.

5 tryps.	+	0	antib.	...	35.85	10 tryps	+	0	antib.	...	71.71
"	+	0.25	"	...	8	"	+	0.25	"	...	41.95
"	+	0.5	"	...	6.6	"	+	0.5	"	...	15.65
"	+	0.75	"	...	6.65	"	+	0.75	"	...	9.6
"	+	1	"	...	6.55	"	+	1	"	...	9.5

The figures obtained illustrate the above rule without any further explanation. In both sets, with large amounts of antibody, a state of neutralisation was arrived at which was not altered on addition of more antibody.

#### DESTRUCTION OF THE ANTIBODY

The antitryptic properties of the serumalbumin are destroyed on boiling, and even on heating to a lower temperature, as shown by Cathcart.<sup>2</sup> At bloodheat it is readily destroyed by 0.2% acetic acid, as shown by following experiments.

The antibody was kept with 0.2% acetic acid at 37°. After intervals of time recorded, samples were taken, from which the acid was removed by dialysis, whereupon the neutralising effect upon trypsin was tried. After the antibody had been acting upon the trypsin for 2 hours at 37°. the amount of trypsin left free was determined with 200 c.c. of casein solution. The results were:—

Without antibody	...	...	...	...	...	55.5
With 5 c.c. intact antibody	...	...	...	...	...	2.1
"	,"	treated with acetic acid for 3 hours				45.9
"	,"	,"	,"	6	,"	48.7

In another experiment, the same antibody was treated with acetic acid for 8 hours:—

Without antibody	...	...	...	...	...	35.95
With antibody treated with acetic acid for 8 hours				...	...	35.45

1. Since this figure lies outside the range of proportionality, it has been calculated by doubling the effect of 5 c.c. of trypsin.

2. *Journ. of Physiol.*, Vol. XXXI, p. 495, 1904.

Therefore the antibody is practically destroyed by 0·2% acetic acid during 8 hours at 37°.

Having thus found that acetic acid readily destroys the antibody and knowing that trypsin is only very slowly attacked by this acid, I thought it important to try whether, in a mixture of trypsin and antibody, which has been kept till the antibody has reached its maximum of effect, the trypsin can be rendered active again by treatment with 0·2% acetic acid at 37°. With this view, I have carried out several experiments, and I have been able to recover the trypsin in none of them. The figures obtained in one of them were as follows :

Without antibody	...	...	...	70
0 hours with acetic acid	...	...	...	3·6
2 "	"	...	...	2·9
4 "	"	...	...	2·5
8 "	"	...	...	2·2

The slight fall in the activity of the mixture might depend upon the destructive influence of the acetic acid upon the trypsin not neutralised by the antibody<sup>1</sup>.

Since some time is always required before all of the antibody is destroyed by the acetic acid, the antibody exercises some neutralising effect even in presence of acetic acid.

#### DISCUSSION OF THE RESULTS

It is generally assumed that enzymes, previous to their action, are in some way attached to the substrate they are able to act upon. On the other hand, antibodies are supposed to form a kind of combination with the substances they neutralise, thus preventing them from being attached to the substrate. My observations, that the amount of trypsin neutralised is larger when the antibody is allowed to act upon the trypsin in absence of a proteid than in presence of the same (p. 474), are quite consistent with these views.

1. With regard to this experiment it may be noted that Morgenroth on treating the compound cobrahaemolysin-antibody and cobraneurotoxin-antibody with HCl was able to recover all of the haemolysin and 50 per cent. of the neurotoxin, *Berl. Klin. Wochenschr.*, 1905, No. 50, and 'Weitere Beiträge zur Kenntniss der Schlangengifte und ihrer Antitoxine'; *Arbeiten aus dem Pathol. Inst. zu Berlin*, 1906.

From the fact that the neutralising effect of the antibody is independent of the dilution (p. 475), one must conclude that the compound which might be formed is not subject to decomposition with water (hydrolysis). There are two other reasons which make me believe that the compound, once formed, does not give off any active trypsin to water. These are :—

- 1.—The antibody neutralises more trypsin the higher the temperature. Nevertheless, if a mixture of trypsin and antibody is kept at a certain temperature till a constant state of neutralisation has been reached, then no trypsin is liberated on lowering the temperature (p. 474).
- 2.—The antibody is readily destroyed by the action of weak acetic acid at  $37^{\circ}$ . If a mixture of trypsin and antibody, having been kept at  $37^{\circ}$  till no more trypsin is neutralised, is digested with weak acetic acid, then no trypsin is rendered active (p. 480).

It must, however, be borne in mind, that the way in which my experiments have been carried out does not exclude the possibility that, after the addition of casein, some trypsin might be transferred from the antibody to the casein. If the antibody readily gave off the trypsin, then the distribution of the trypsin would become the same as if the casein had been present from the very beginning. The fact that this is not the case shows that the trypsin, when once attached to the antibody, is not readily given off to casein. Nevertheless, it cannot be entirely excluded that some change may take place, and a transport of trypsin from the antibody to the casein might, perhaps, account for the fact that it has been found impossible completely to neutralise trypsin by means of the antibody (p. 476).

Since trypsin and its antibody undoubtedly, in more than one respect, behave in a way similar to some toxins and their antitoxins, it might be useful to consider the question, whether the theories put forth for the explanation of the relationship between the latter might apply to the trypsin and its antibody.

According to Ehrlich's theory,<sup>1</sup> the compound toxin-antitoxin is

1. *Fortschritte d. Med.*, 1897; *Werthbestimmung des Diphtherieheilserums*, 1897.

merely a chemical one, like the combination between a strong base and a strong acid. This theory, therefore, involves that the two constituents should combine till one of them is completely bound up and that they by proper means can be separated again. Moreover, Ehrlich assumes that some toxins (*e.g.* diphtheria toxin) are made up of constituents of different toxicity and different avidity to the antibody. By aid of this theory it might be explained that a large amount of trypsin can be neutralised without leaving any antibody free, but the fact that on further addition of antibody the fluid may contain free trypsin and free antibody at the same time this theory does not account for, unless one assumes that trypsin is made up of different constituents. Furthermore, the fact that I have not been able to separate the two constituents of the trypsin-antitrypsin compound is not in favour of Ehrlich's theory.

Arrhenius and Madsen<sup>1</sup> assume that toxin and antitoxin behave like a weak base and a weak acid. Therefore, the compound is subject to hydrolysis, and there always exists some free toxin and some free antitoxin at the same time. Moreover, their theory requires that the compound should be reversible, *i.e.*, the equilibrium ought to be the same under the same conditions, no matter in which way they have been arrived at. This theory seems to be inconsistent with the fact that I have been able to neutralise more than three-quarters of the trypsin without any antibody remaining free in the fluid. In addition, the compound has not been found to be reversible in my experiments (p. 481).

Bordet has suggested that the antibodies be capable of combining with varying amounts of toxin, and consequently he holds that the combination toxin-antitoxin be not a chemical one. He assumes that the toxin is taken up by the antitoxin, very much as a dye is by a tissue. This view takes into account the fact that the agents, being colloids, may, perhaps, not follow the laws of genuine solutions.<sup>2</sup> This view has more recently been advanced by Nernst<sup>3</sup> and by Craw.<sup>4</sup>

1. *Festschrift ved invielsen af statens seruminstitut*, Copenhagen, p. 56, 1902.

2. *Ann. Inst. Pasteur*, Vol. XVII, p. 161, 1903.

3. *Zeitschr. physik. Chem.*, Vol. XLVII, p. 54, 1904.

4. *Proc. Roy. Soc.*, Vol. LXXVI, B, p. 179, 1905.

Trypsin and antitrypsin, being colloids, do not form any real solutions with water, but only suspensions of larger or smaller particles. Since very little is known as to the ways in which suspended particles might act upon each other, I have tried to throw some light upon this subject by investigating how charcoal acts upon trypsin, thus substituting charcoal for the tryptic antibody in my above experiments. The results are laid down in the following paper and the further discussion of the subject must be postponed till the account of these experiments has been given.

The results of my previous investigations on trypsin and antitrypsin are given on p. 474. The conclusions of the investigations in this paper are as follows :—

1.—The amount of trypsin neutralised by the antibody is independent of the dilution.

2.—The antibody can be completely saturated by using a sufficient amount of trypsin ; on the other hand it has been found impossible under the conditions used, completely to neutralise trypsin by an excess of antibody.

3.—The amount of trypsin neutralised by a certain amount of antibody is not constant, a small amount of antibody neutralising relatively more trypsin than a larger.

4.—The antibody can be completely destroyed by 0.1—0.2% of acetic acid at 37° for about 8 hours.

5.—When trypsin has been neutralised by the antibody, it has been found impossible by the means tried to render it active again.

AN ANTITRYPTIC EFFECT OF CHARCOAL AND A  
COMPARISON BETWEEN THE ACTION OF CHARCOAL  
AND THAT OF THE TRYPTIC ANTIBODY IN THE  
SERUM.

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By chance I recently observed that charcoal, when added to a digesting fluid of trypsin and casein, produces a very marked diminution of the tryptic effect. There can hardly be any doubt that this depends upon the trypsin being taken up by the charcoal, thus being prevented from acting upon casein. It is a well known fact that charcoal readily condenses gases on its surface, and likewise takes up colouring matters from solutions. That other solid substances and especially hydrogels, have got the power of taking up crystalloids from their solutions has been pointed out by several investigators (v. Bemmelen,<sup>1</sup> Schmidt,<sup>2</sup> Biltz,<sup>3</sup> Bayliss,<sup>4</sup> and others). This process has been termed adsorption (Du Bois Reymond, Ostwald).

As to the laws of adsorption very little is known. Henry's rule, which has been found to be valid for the distribution of a soluble substance between two fluids, which implies that the ratio between the concentration in the two solvents is a constant ( $\frac{C_1}{C_2} = k$ ), when equilibrium has been reached, does not apply to the adsorption. In the case of adsorption, a state of equilibrium seems to be arrived at sooner or later ; if  $C_{ads}$  is the concentration in the solid substance and  $C_a$  the concentration in the fluid, then  $\frac{C_{ads}}{C_a}$  is not a constant, but diminishes as the total amount of the originally dissolved substance is increased. This means that the solid takes up relatively more from

1. *Zeitschr. anorg. Chem.*, Vol. XXIII, p. 321, 1900.
2. *Zeitschr. physik. Chem.*, Vol. XV, p. 60, 1894.
3. *Chemiker Zeitung*, Vol. XXIX, No. 25, p. 325, 1905.
4. *Bio-Chem. Journ.*, Vol. I, p. 175, 1906.

a dilute than from a concentrated solution. The way in which the ratio  $\frac{C_{ads}}{C_a}$  varies with the total amount of dissolved substance is different in different cases, and no general rule has been found. As to other factors which influence the adsorption v. Bemmelen points out the following:—(1) the adsorbing substance, (2) the solvent, (3) the substance to be adsorbed, (4) the state of its molecules, (5) the temperature, without being able to give any general rules as to their influence.

In the cases where the adsorption of colloids has been investigated, the influence of the amount of colloid upon the ratio  $\frac{C_{ads}}{C_a}$  has been found to be roughly the same as for crystalloids (Bayliss). In addition, v. Bemmelen observed that the adsorption of colloids (e.g.,  $As_2S_3$ ) may sometimes be complete. Bayliss found that the total amount of congo-red taken up by paper is less the higher the temperature, and he also observed that the congo-red can be completely removed from the paper by repeated extraction with water.

Adsorption of enzymes by solids, and particularly by charcoal, has been observed by Glaessner,<sup>1</sup> Dauwe,<sup>2</sup> and others, but no investigations as to the conditions and quantitative relations of the adsorption have so far been published.

My experiments on the adsorption of trypsin by animal charcoal were carried out with a view to make out whether there exists any analogy between the action of the charcoal upon trypsin and the action of the tryptic-antibody in the normal serum. The animal charcoal used had been prepared from bones, and the phosphates had been removed by repeated heating with HCl. The trypsin had been obtained as given in a previous paper<sup>3</sup>. The digestive power of the trypsin was estimated as described in the same paper, and the figures given represent the number of cubic centimetres of decinormal acid required to neutralise the ammonia obtained from equal volumes of the tannic acid filtrates.

1. Hofmeisters *Beiträge*, Vol. I, p. 1, 1902.
2. *Ibid.*, Vol. VI, p. 427, 1906.
3. Hedin, 'Observations on the action of trypsin,' *Journ. of Physiol.*, Vol. XXXII, p. 486, 1905.

## ADSORPTION WITHOUT THE PRESENCE OF PROTEID

In the first place it was found that the presence of a proteid (*e.g.* casein) materially influences the process of adsorption, and therefore some experiments were carried out in which only the small amount of proteid contained in the trypsin solution was present during the process of adsorption. Thereupon the charcoal was filtered off, and the digestive power of the filtrate was tested with casein.

*Experiment I.*—Two mixtures containing, the one 2 gr. charcoal + 80 c.c. tryps. (A), and the other 0.1 gr. charcoal + 80 c.c. tryps. (B), were kept at 20° as recorded below. Thereupon, equal volumes of the filtered samples were made to act upon 50 c.c. of 2.5% casein solution at 37° for 24 hours. 25 c.c. of 10% tannic acid solution were added, and 50 c.c. filtrate were taken for each estimation. The trypsin used for each estimation would, without previous treatment with charcoal and with a sufficient amount of casein, have given the result 240.

	A.	B.
1 hour	0	55.4
4 hours	0	53.75
24 , , ...	0 ...	46.75
48 , , ...	0 ...	43.5

The influence of the amount of charcoal is evident, the adsorption in A with 2 gr. of charcoal being complete after one hour, and in B with 0.1 gr. equilibrium being reached in 24 hours. The small drop observed after the expiration of this time may depend upon destruction of enzyme, as shown by the fact that samples of the same enzyme having been kept without charcoal for two days at 0°, gave 38.6, and at 20° 34.2.

The influence of the temperature was examined by putting the mixture B (after 48 hours at 20°), at 37° for 24 hours. The result was :

After 48 hours at 20°	...	...	...	43.5
After 48 hours at 20° and 24 hours at 37°				2.3

Therefore, if the temperature is raised after equilibrium has been reached at a low temperature, then more trypsin is adsorbed.

*Experiment II* shows the influence of the temperature and of the time, low temperatures being used in order, as far as possible, to prevent destruction of the trypsin. Samples of the same mixture were kept at 0° and at 20°, as given below. Then the filtrates were tested as above. Without charcoal 120.

		0°		20°
24 hours	...	22.3	...	4.5
48 "	...	7.95	...	0.2

The experiment shows that more trypsin is adsorbed at the higher temperature. The equilibrium was not reached in 24 hours.

*Experiment III* was carried out at 20°, and only the amount of charcoal was varied.

0.5 gr. charcoal	...	0	0.05 gr. charcoal	...	25.75
0.1 gr.	„	3.75	Without	„	750

This experiment verifies the rule that  $\frac{C_{ads}}{C_f}$  diminishes as the total amount of enzyme is increased, or (which comes to the same) as the amount of charcoal is diminished. A consequence of this rule is, that when the amount of trypsin is the same and the amount of charcoal is diminished, then the charcoal approaches by degrees a state of saturation, where no more trypsin is adsorbed. I have been able to verify this by keeping mixtures of charcoal and trypsin at 20° for 24 hours, whereupon fresh trypsin was added to equal volumes of the fluids before (A) and after (B) filtration. If the charcoal is not saturated, then A gives less digestion than B, and at saturation both give the same result.

0.025 gr. charcoal	...	...	(A. 28
		...	(B. 36.3
0.0125 gr.	„	...	(A. 49.8
		...	(B. 52.65

In this experiment, 100 c.c. of casein solution was added as substrate. The result shows that saturation was nearly reached in the mixture with 0.0125 gr. charcoal.

The above results are remarkable as showing how readily charcoal effects a complete adsorption of trypsin. Similar observations have

been made before by v. Bemmelen (page 485), but to my knowledge complete adsorption has not been observed for crystalloids.

According to the above experiments, more trypsin is adsorbed the higher the temperature. In the case of the adsorption of congo-red by paper, the influence of the temperature is the reverse (Bayliss, p. 485).

#### INFLUENCE OF THE PRESENCE OF THE CHARCOAL DURING THE DIGESTION

In all the above experiments, the charcoal used for the adsorption was removed before the casein was added. The result of the digestion becomes somewhat different if the charcoal is not removed, as proved by the following experiment.

*Experiment IV.*—The trypsin and the charcoal were allowed to act upon each other as recorded, then equal volumes of the fluids were digested with 50 c.c. of casein solution in set A, after filtering off the charcoal, and in set B, in the presence of the same.

		A.	B.
0.5 gr. charcoal	24 hours at 37°	...	0
0.25 "	" 37°	...	0
0.5 "	" 17°	...	0
0.5 "	48 hours at 37°	...	0
0.25 "	" 37°	...	0

The amount of trypsin rendered inactive by the charcoal was very considerable, since the trypsin used for each digestion, without charcoal and with an excess of casein, would have given the result 960. No sample of set A showed any tryptic effect, which implies, that before the addition of the casein the adsorption was complete in all samples. The digestion taken place in the samples of Set B, therefore, must depend upon the casein being able to deprive the charcoal of a small amount of the trypsin adsorbed. This is confirmed by the following experiment.

*Experiment V.*—An amount of trypsin, which corresponded to the effect 2400, was kept with 0.5 gr. charcoal at 37° for 24 hours.

Then the adsorption was complete, 5 c.c. of filtrate, in 24 hours,

with 50 c.c. of casein solution, giving the effect 0·7. The charcoal was thoroughly washed and digested with 50 c.c. of casein solution. The effect was 30·6.

That the trypsin is attached to the casein before the digestion, is proved by the following experiment.

*Experiment VI.*—A mixture of 0·5 gr. charcoal + 80 c.c. tryps., was kept at 20° for five hours. The adsorption was then complete. To 50 c.c. of the mixture 300 c.c. of casein solution at 37° were added, and the whole was kept at 37° for one hour. Then part of the mixture was repeatedly filtered in order to remove the charcoal. 50 c.c. of the casein-charcoal-trypsin mixture and 50 c.c. of the filtrate were digested with the following result :—

With charcoal	...	...	...	...	25·1
Without „	...	...	...	...	23·2

Another experiment with other proportions gave

With charcoal	...	...	...	...	65·8
Without „	...	...	...	...	64·4

The experiments show that all the trypsin available had been transferred from the charcoal to the casein during one hour at 37°.

Therefore, the casein undoubtedly is capable of taking back some of the trypsin already adsorbed by the charcoal. The amount taken back in the above experiments was 1-15% of the amount originally adsorbed. The amount of trypsin rendered active by addition of casein after complete adsorption by charcoal depends upon

- 1.—The amount of charcoal used, less trypsin being rendered active the more charcoal present.
- 2.—The temperature at which the adsorption has taken place, less trypsin being rendered active the higher the temperature.
- 3.—The time of interaction between the trypsin and the charcoal, less trypsin being rendered active the longer the time.

This is all borne out by Experiment IV.

The influence of the temperature was tried in another experiment, where lower temperatures were used, in order, as far as possible, to prevent destruction of enzyme.

*Experiment VII.*—Two equal mixtures were kept for 48 hours, the one at 0°, the other at 20°, whereupon the digesting power was tested after filtering off the charcoal (A) and in the presence of the same (B). The amount of trypsin used, would, without treatment, with charcoal, have given the effect 93.

		A		B
48 hours at	0°	...	0	...
48	20°	...	0	...

According to the above investigations, the process, by which trypsin is rendered inactive by charcoal, can be divided up into two different stages: (1) the *taking-up*, or adsorption of the trypsin by the charcoal, and (2) the *fixation* of the same. The amount of trypsin taken up by the charcoal is represented by the difference in digesting power between the trypsin not treated with charcoal, and the filtrate after treatment with charcoal. The process of fixation can most conveniently be studied in experiments with rather a large amount of charcoal, in which case all the trypsin very soon is taken up, whilst the fixation is still in progress. In such cases the amount of trypsin taken up and not fixed is obtained by direct estimation of the digestive power of the mixture.

If very little charcoal is used, then both processes come to a standstill before all trypsin is rendered inactive. This shows that the trypsin is not merely destroyed, because in that case the process would not finish till all trypsin were destroyed. It is very difficult to form a clear view of what is taking place. At present I am inclined to think that the trypsin is attached to the surface of the charcoal by molecular attraction, and subsequently proceeds further into the charcoal by diffusion, thus, by degrees, becoming inaccessible for the proteid. As long as the trypsin is attached to the surface of the charcoal, or merely adsorbed, it can be transferred to added casein, but not when it has entered deeper into the same. This view would account for the fixation going faster the more charcoal is present, *i.e.*, the larger the surface through which the diffusion takes place. It would not be difficult to conceive that a process of diffusion would depend upon the temperature and the time, as found above, for the

process of fixation. In this way it would be easy to understand that only part of the trypsin taken up by the charcoal can be rendered active by addition of casein.

As pointed out, charcoal, if present in sufficient amount, adsorbs trypsin completely, leaving no trypsin in the filtrate when filtered off. On the other hand, if casein is added to the mixture of charcoal and trypsin before filtration, then a considerable amount of the trypsin already adsorbed appears in the filtrate together with the casein. This fact affords a very strong support for the view that trypsin is attached to proteids previous to its action, and it does not seem unlikely that this is a process of adsorption. This would be quite consistent with the fact that proteids do not form any real solutions with water, but suspensions of larger or smaller particles. If the taking-up of trypsin by proteids is a process of adsorption, then it might be expected that the proteids, if present in sufficient amount and in absence of other adsorbing substances, could, like charcoal, completely adsorb the trypsin. In a previous paper I have pointed out that complete combination of the trypsin is required for the explanation of the following two observations :—

- 1.—The effect of trypsin upon certain proteids is independent of the dilution, and
- 2.—The effect is the same after the same number of trypsin time units.<sup>1</sup>

#### COMPARISON OF THE NEUTRALISING POWER OF CHARCOAL WITH THAT OF THE TRYPTIC ANTIBODY IN THE SERUM

From the above experiments it is evident that charcoal exercises a very marked checking influence upon the action of trypsin, which influence is very much like the effect produced by the tryptic antibody contained in normal serum. In the case of charcoal, the process of neutralisation can easily be distinguished from the process of digestion, by filtering off the charcoal before the proteid to be digested is added. In the case of the antibody, the two processes cannot be strictly

1. *Journ. of Physiol.*, Vol. XXXII, p. 480, 1905.

distinguished, because the neutralising substance cannot be removed. In order to compare the effect upon trypsin of charcoal and of antibody, one, therefore, has to carry out the digestion in the presence of the neutralising substance in both cases. Investigations of that kind I have previously carried out as far as the antibody is concerned.<sup>1</sup> Corresponding observations with charcoal can be obtained from Experiments IV and VII. Some additional observations are given below. Investigations have been carried out with reference to:—

- 1.—The time of interaction of trypsin and the neutralising substance.
- 2.—The temperature.
- 3.—The dilution.
- 4.—The saturation of the neutralising substance with trypsin, and
- 5.—The complete neutralisation of trypsin.

From the above experiments it follows that the *amount of trypsin neutralised is larger, the longer the time and the higher the temperature.* Complementary conclusions can be drawn from the following experiments.

*Experiment VIII.*—Mixtures of 1 gr. charcoal + 20 c.c. tryps. were kept as given below. Then the digesting power of the mixtures was determined with 50 c.c. of casein solution at 37° for 24 hours.

		17°		37°
3 hours without charcoal	...	43.65	...	43.4
5 min. with charcoal	...	30.5	...	22.9
30 min.	”	25.5	...	lost.
1 hour	”	21.4	...	9.2
3 hours	”	16	...	2.95
3½ hours	”	14.35	...	2.15

Equilibrium was not reached in three hours.

With another enzyme, the following results were obtained.

		20°		37°
18 hours	...	40.6	...	11.85
19 ”	...	39.9	...	9.8

1. See page 474.

The amount of enzyme neutralised was very considerable, since the trypsin used, with an excess of casein, would have given the figure 470. One sample, which had been kept at 37° for 18 hours (corresponding to 11.85), was kept at 20° for 24 hours, and gave then the result 9.6. Another sample, which had been kept at 20° for 18 hours (corresponding to 40.6), lost in digesting power during eight hours at 37° (result = 25.1).

This experiment, and others which have not been recorded, show that a very long time is wanted for reaching the state of equilibrium, where no change takes place. This makes it very difficult to decide whether a diminution in activity be due to adsorption or to destruction of enzyme. Considering the enormous amount of enzyme rendered inactive by the charcoal the fall after 24 hours is however rather insignificant, and I think one is justified in saying that equilibrium is arrived at, although very slowly.

*Experiment IX.*—In order to find out whether the *dilution* has any influence upon the amount of trypsin rendered inactive by charcoal, specimens were made up as follows:—

0.05 gr. charcoal + 20 c.c. trypsin	...	...	...	A
0.05	"	+ 20 c.c. H <sub>2</sub> O	...	B
0.05	"	+ 40 c.c. H <sub>2</sub> O	...	C.

After 24 hours at 20°, 40 c.c. of water were added to A, and 20 c.c. to B, whereupon the digestive power of the specimens was estimated as usual. The result was:—

$$A = 78.4 \quad B = 80.3 \quad C = 80.5$$

and consequently the *dilution had no influence*. Control specimens, which were filtered before the casein was added, showed that the adsorption was not complete.

*Experiment X.*—Whether charcoal can be *saturated* with trypsin was tried as follows. 0.01 gr. charcoal + 50 c.c. tryps. was kept at 20° for 24 hours, and then at 37° for 24 hours. The digestive power was determined with an excess of casein (20c c.c.), in which case the results obtained are proportional to the amount of active trypsin present.<sup>1</sup>

1. *Journ. of Physiol.*, Vol. XXXII, p. 471-474, 1905.

Three estimations were carried out, namely, of 5 c.c. of the above charcoal-trypsin mixture, of 5 c.c. of this mixture in the presence of 1 c.c. of trypsin, not previously acted upon by charcoal, and of this amount of trypsin alone. The sample, with mixture and trypsin, was kept at  $37^{\circ}$  for one hour before the casein was added. The results were :—

5 c.c. of mixture	...	...	...	12.1
5 c.c. , ,	+ 1 c.c. of tryps.			35.3
1 c.c. of trypsin	...	...	...	23.4

Therefore, the effect of the trypsin, by itself, (23.4) was the same as the effect of the trypsin in the presence of the charcoal-trypsin mixture ( $35.3 - 12.1 = 23.2$ ), which proves that the charcoal in the mixture had not affected the trypsin, and that the *charcoal was saturated with trypsin* before the fresh trypsin was added.

As to the question, *whether trypsin can be completely neutralised by charcoal*, it is sufficient to refer to the above investigations, according to which it is very easy completely to remove all trypsin from a solution, by using a large amount of charcoal and filtering it off before the casein is added, but that *always some tryptic effect has been observed, when the digestion has been carried out in the presence of the charcoal which had absorbed the trypsin*.

The above results, obtained with trypsin and charcoal, as compared with corresponding results obtained with trypsin and its antibody in the serum,<sup>1</sup> show that in all respects tried, there exists a very close agreement between charcoal and the antibody, with regard to their effect upon trypsin. In consequence of this agreement, I am very much inclined to believe that the neutralisation of the trypsin is brought about in the same way in both cases. As I have pointed out above, the neutralisation of trypsin by charcoal implies, not only the *adsorption* or the taking-up of trypsin by the charcoal, but also the *fixation*, or the process by which the trypsin is rendered inaccessible for proteids. In all my experiments with charcoal some trypsin has remained merely adsorbed, and therefore transferable to added casein

1. See page 483.

and active. Accordingly, it is only reasonable to think that the same might have been the case in experiments with the antibody, and, in my opinion, this is a very acceptable explanation of the fact, that it has been found impossible completely to neutralise trypsin by aid of the antibody.

### CONCLUSIONS

1.—The process of neutralisation, exercised by charcoal upon trypsin, can be divided in two consecutive stages :—

(a) The taking-up, or *adsorption* of the trypsin by the charcoal.

A complete adsorption of the trypsin can easily be effected by using a sufficient amount of charcoal. The trypsin merely adsorbed is readily transferred to added casein.

(b) The *fixation*, or the process by which the trypsin is rendered inaccessible for added casein. The amount of trypsin fixed is larger, the larger the amount of charcoal, the longer the time of interaction and the higher the temperature.

2.—The action of charcoal has been found to agree with that of the tryptic antibody in all respects tried, and therefore the neutralising effect, in all probability, is brought about in the same way in both cases.





